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# REVERSIBILITY OF MORPHOGENETIC PROCESSES IN BURSARIA

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THIRTY-EIGHT FIGURES

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## I. INTRODUCTION

A review of the extensive literature on structural regulation in organisms will show that the results have been obtained and the theories to explain the phenomena have been formulated from facts derived from studies on cell groups such as whole individuals, organs or tissues. Little attention has been given to the changes which must take place in the individual cell during regulation. Few attempts have been made to contribute an answer to such questions as: Just what changes take place in

cells when by division or otherwise, they replace lost cells which may have had a different structure and function? Is there a direct transformation of one type of cell or differentiated part of a cell into another, and if so how is this transformation brought about? What is the nature of the physico-chemical changes which constitute structural regulation in the cell?

The following facts derived from a careful study of over 3500 individuals of the ciliate protozoan *Bursaria* sp. are presented with the hope that they may help to throw light on these questions and possibly point out what is the general nature of the process which brings about dedifferentiation.

## II. THE NORMAL PROCESS OF DIFFERENTIATION AND DEDIFFERENTIATION

### 1. *During cell division*

In any structurally differentiated cell which does not possess any plane of symmetry it is necessarily true that cell division would result in the formation of dissimilar parts unless some process took place through which the daughter cells became alike. The more striking cases of this kind occur among the more differentiated types of protozoa Wallengren ('01). But even in this group it is difficult to find examples where the process of structural regulation at division can be followed with any degree of ease. *Bursaria*, a heterotrichous ciliate of fairly common occurrence, is of sufficient size and complexity of organization to permit one to follow with relative clearness the change in structure which occurs during cell division, regeneration and certain other processes.

Figures 1 to 6 inclusive, of plate I, show ventral views of *Bursaria* in different stages of division.<sup>1</sup> The closure of the gullet and peristome results in a complete disappearance of the membranelles lining the dorsal wall of the gullet (figs. 2 and 3, *m*), and other structures such as the ciliated ridge or septum (fig. 1, *s*). The changes may be better seen in cross sections from a plane

<sup>1</sup> I wish to express my indebtedness to Miss Helen Sanborn for the drawing of all the figures except those in plate 4.

midway between the anterior and posterior ends of the individual. Figure 18 is a cross section of a fully differentiated normal individual as shown in figure 1 or figure 6. Figure 16 is a cross section through the middle of one of the daughter cells at the stage in division shown in figure 4. The membranelles, septum and gullet have disappeared or *dedifferentiated* leaving only the anlage (fig. 16, *a*). Figure 17 is a cross section from the same level in the cell as the sections shown in figures 16 and 18, but at a stage in differentiation after division shown in figure 5. Differentiation proceeds by inward movement and expansion of the anlage to form the gullet. As the gullet deepens the membranelles appear on its dorsal wall; the septum with its long slender cilia appears and the peristome opens. The real mouth lies at the inner end of the curved gullet (fig. 1, *o*). The action of this feeding mechanism has been described in connection with the reactions of *Bursaria* to food (Lund, '14).

Differentiation takes from thirty minutes to an hour for its completion, or about the same length of time as the process of dedifferentiation, depending upon conditions such as temperature, mechanical stimulation, medium, etc. Dedifferentiation during normal division is never complete, a trace of the gullet and peristome always remaining. This I have called the anlage of the gullet. Figure 19 is longitudinal sagittal section through a normal fully differentiated individual such as shown in figure 1.

## 2. *Before and after encystment*

The anlage of the gullet and peristome completely disappears before encystment takes place. The cell becomes spherical or very nearly so, showing that internal tensions are more nearly equalized than at division. The process of dedifferentiation proceeds farther than at cell division. Figure 7 shows the cell as a dedifferentiated ciliated sphere. In this condition as well as at division (figs. 3 and 4), the cell is strongly thigmotactic, sticking to the bottom of the watch glass or surface film. During encystment (fig. 8), the cilia melt away. The protoplasm apparently loses water, reduces in volume and becomes more dense,

as indicated in figures 7, 8 and 9. A clear body of hyaloplasm appears at the cyst hilum (fig. 8, *r* and *h*; fig. 9, *h*). The hilum is a thin part of the cyst which perhaps is readily permeable and through which chemical stimuli may act upon the clear cytoplasmic body lying beneath it.<sup>2</sup>

Some of these changes during encystment have been partly described and figured by Brauer, '86.

Cells resulting from division may lose the anlage of the gullet and then encyst without first fully differentiating. The arrows between figures 5, 6, and 7 indicate the possible sequence in the process. When encystment takes place, differentiation of the protoplasm is preceded by increase in size, owing to absorption of water. The upper arrows between figures 9, 8, and 7 refer to the reversal of the morphogenetic processes which take place in the protoplasm and not the cyst.

### 3. *Spontaneous dedifferentiation and differentiation*

One striking fact about the morphogenetic process in *Bursaria* is that dedifferentiation of a fully differentiated individual often takes place without any apparent external cause initiating the change. Within an hour or several hours, opening of the peristome and gullet takes place with return to the fully differentiated condition (figs 1 and 2). The extent of dedifferentiation and closure of gullet is not the same in all these cases. I have histories of individuals in which this process was repeated two and three times during the course of two to ten hours when they were kept in watch glasses containing tap water. Figure 20 is a horizontal longitudinal section of a spontaneously dedifferentiated normal individual. Dedifferentiation has proceeded in this case a little farther than in that shown in figure 2, for all trace of membranelles is absent, leaving only the anlage of the gullet (fig. 20, *a*),

<sup>2</sup> All cysts are not of the same size, as shown by measurements of cysts from two sets of individuals; one set was fed egg yolk and the other was not fed. Cysts from the well fed individuals were larger than cysts from those of the starved set. Abnormal cysts are often formed.

From what precedes the following facts should be noted: (1) Dedifferentiation followed by differentiation is a normal process and at least from a morphological standpoint may be considered reversible. (2) It is a distinct morphogenetic response which occurs under a number of different conditions and to different extent.

### III. INITIATION OF DEDIFFERENTIATION AND DIFFERENTIATION

#### *1. By cutting into two or more parts*

A large number of experiments were made in each of which records of cut pieces from sets of twenty-five to one hundred individuals were taken at intervals varying from an hour to twelve hours. In most cases the record of the condition of each piece were continued until death, encystment or starvation of the regenerated pieces ended the experiment. The individual to be cut was placed in a small drop of water on a slide and cut, under the low or high power of the binocular, with a fine pointed curved scalpel. Anterior and posterior halves or right and left halves are quite readily obtained in this way with a little practice. The pieces were placed in separate watch crystals or depression slides containing tap water and kept in moist chambers. Unfortunately a large number of the experiments designed to yield quantitative data on such questions as the effect of size of nuclear piece on regeneration, rate and capacity for regeneration of pieces cut at different ages, etc., proved to be useless, except in so far as they showed that the results depended largely on the state of nutrition and previous history of the cell. It has been impossible as yet to thoroughly control these in *Bursaria* and hence the results did not show any definite uniformity.

The results as far as they concern the normal process of regulation of anterior and posterior pieces are summarized plate I, figures 10 to 15 inclusive. Figure 10 is partly schematized for the sake of clearness by showing the cut surface as it would appear if it did not close. The other figures are close representations of the actual appearance of regenerating pieces at various stages of dedifferentiation and differentiation.



Dedifferentiation of an anterior or posterior half (figs. 11, 12, 13) before it again differentiates, is more complete than during normal division (cf. figs. 13 and 4). The pieces assume a spherical shape before differentiation takes place. Generally all trace of the mouth and gullet disappears. It seemed to be true that larger pieces, for example anterior or posterior two-thirds, did not dedifferentiate as completely as the smaller pieces, for usually in the larger pieces traces of the oral apparatus never completely disappeared.

A fully dedifferentiated piece may either encyst or differentiate to a normal individual. Encystment often takes place after a piece has differentiated and formed a normal individual, but in every case dedifferentiation of the regenerated piece occurs before encystment. The arrows between figures 13, 14, 15 and 7 indicate the possible sequence. These results are based upon individual records of hundreds of cut pieces. Right and left halves formed by cutting the individual lengthwise undergo dedifferentiation and differentiation closely similar to anterior and posterior halves.

The nuclear apparatus consists of about 9 to 15 small micronuclei (not shown) and one large macronucleus (*mn*). Pieces without a part of the macronucleus never regenerate, but regeneration is possible in pieces which contain only a small part of the macronucleus. The size of the macronuclear piece included seemed to have no effect on the rate of regeneration, for two halves of an individual, one containing a small and the other a very large piece of the macronucleus usually regenerated in about the same length of time,—as shown by the records. All pieces which regenerate, presumably have one or more micronuclei. This point would be difficult to decide because of the small size of the micronuclei in *Bursaria*.

## *2. By mechanical injury without removal of parts of the cell*

If the anlage of the gullet of any dedifferentiated individual is injured by tearing it with a needle and if the injury is not too great the cell may continue to differentiate but the wound persists as a more or less abnormal part of the gullet which other-

wise is normally differentiated. This abnormal part may remain as such for many hours unless dedifferentiation leading to redifferentiation or encystment takes place. If the injury to the anlage is greater, dedifferentiation occurs, so as to eliminate all visible traces of the anlage, with subsequent differentiation to fully normal form. From observations on the numerous individuals injured in this way, it seems that considerable variation occurs as to how great the mechanical injury to the anlage must be before dedifferentiation sets in. The experiment is most easily carried out on a dividing individual. From this it appears that dedifferentiation proceeds only to the extent necessary for complete duplication of the structure. One might crudely compare the process to the rebuilding of a damaged house; if the old foundation is uninjured or only slightly injured it may be used again for supporting a new frame; while if the foundation is seriously injured it also must be rebuilt. We see here the essentials of the phenomenon of localization as found in the development of the egg of many different metazoa, for from the above experiments there appears to exist in the anlage of the gullet a definite part which corresponds to a definite structure in the fully differentiated gullet.

#### IV. DEDIFFERENTIATION AND DIFFERENTIATION IN HETEROMORPHS

Throughout the work a total of about seventy-five abnormal individuals was observed. A few of these were studied carefully, and observations made from time to time in order to follow their histories. Very close observations on such individuals were difficult to make because of continuous movements and the impossibility of manipulating them for observation under higher powers. This nearly always resulted in loss or injury to the specimen whose history was desired. The observations were made under low and high powers of the Leitz binocular and also with the low power of the compound microscope. Drawings were necessarily made free hand, but care was taken to obtain approximately correct proportions. Some of the individuals

most carefully studied are shown in figures of plates IV and V. They will illustrate the observed phenomena.

*1. Reversal of polarity in heteromorphic individuals from cut pieces*

The individuals described in this section developed from equal anterior, posterior, right or left halves. They are shown in plate 4. The following is a transcript of brief notes made at intervals after cutting, giving the history of the pieces. The arrows in the figures indicate the direction of the ciliary currents over the body and in the gullet.

*Experiment 7a.* Fifty individuals cut longitudinally. Three of the 100 pieces became heteromorphic and are described (plate 4).

Figures 21a' and 21b are from halves of same individual. History. Six hours after cutting it was irregular. Twelve hours after cutting, triangular, apparently several pieces of nucleus fused; drawn (fig. 21a). Twenty-four hours, heteromorphic, drawn (fig. 21a'). Thirty-six hours, dead.

Figure 21b. History. Six hours, dedifferentiated. Twelve hours elongated, very abnormal. Twenty-four hours heteromorphic, drawn (fig. 21b). Thirty-six hours, active, protoplasm clear (this was due to starvation). Macronucleus all withdrawn into larger individual; ciliary currents same as at twenty-four hours. The smaller individual was narrower than in figure 21b. Forty-eight hours, thick cyst formed from large individual. Small, spindle-shaped, dedifferentiated, non-nucleated active piece present in watch glass. This was the isolated remains of small individual of the heteromorph at twenty-four hours. Sixty hours; small piece dead.

Figure 22. History. Six hours, small piece of macronucleus present, dedifferentiated; a trace of an oral groove at each end; beat of cilia indicate heteromorphic condition, drawn (fig. 22). Twelve hours; presence of trace of oral grooves doubtful but ciliary currents and shape as at six hours. Twenty-four hours; irregular, not active. Thirty-six hours, pear shaped, active. Forty-eight hours, cyst, dead. The sister piece regenerated after thirty-six hours to a perfectly normal individual which lived eighty-four hours.

*Experiment 5.* Fifty individuals cut longitudinally. Two of the 100 pieces became heteromorphic. These two pieces were halves of the same individual.

Figure 23a. Twenty-four hours after cutting it was heteromorphic. One large gullet and mouth nearly normal. The other end had a small oral groove with no membranelles, ciliary currents opposite, drawn. Forty-eight hours, normal, all ciliary currents in same direction. The

larger end of figure 23a had become dominant. Seventy-two hours, one oral groove, partly closed. Ninety-six hours, dead.

Figure 23b. Twenty-four hours; heteromorphic (shown by ciliary currents). A rudimentary mouth at each end, oral groove continuous, nucleus present. The action of the cilia reminds one strongly of the cilia of *Paramecium* when under the influence of a direct electric current. Forty-eight hours, dead.

*Experiment 3.* Forty individuals cut into anterior and posterior halves. Two out of the 80 pieces became heteromorphic. At intervals the regenerated pieces were fed protozoa from a wild culture not containing *Bursaria*. This in part accounts for the long life of the regenerated pieces.

Figure 24. Six hours; almost fully regenerated. Twenty-four hours; perfectly normal, and continued to remain normal up to ninety-six hours. At one hundred and twenty hours it was abnormal, in shape of a pyramid. One hundred and forty-four hours and one hundred and sixty-eight hours still abnormal. One hundred and ninety-two hours heteromorphic; drawn (fig. 24). At two hundred and sixteen hours it was nearly normal, ciliary currents in same direction, one gullet quite well differentiated. Two hundred and forty-eight hours, dead.

Figure 25. Six hours; regenerated to normal, twelve hours; perfectly normal. Forty-eight hours; perfectly normal. Seventy-two hours; normal but with a notch at posterior end. One hundred and twenty hours, normal, large. At one hundred and ninety-two hours it had divided into four; two of these were dedifferentiated, these were removed from the watch glass. The other two formed an equal heteromorphic pair, drawn (fig. 25). Arcellae had been ingested by both individuals of the heteromorph. Two hundred and sixteen hours, two separate, dedifferentiated individuals resulting from separation of heteromorph. Two hundred and forty hours, dead from starvation.

*Experiment 7b.* Fifty cut crosswise into two equal anterior and posterior halves. One out of the 100 pieces became heteromorphic.

Figure 26. Twelve hours, partly dedifferentiated, oval, with spine present. Twenty-four hours, heteromorphic, drawn (fig. 26). Fed with other protozoa. Thirty-six hours, normal, spine had become constricted off at base and the gullet at the base of the spine had dedifferentiated and disappeared. The ciliary beat on the posterior end was now reversed to agree with that of the larger end. Forty-eight hours; perfectly normal; the free cytoplasmic piece formed from the spine had no macronucleus, was active, but had decreased in size. Sixty hours, perfectly normal, feeding. The detached spine was small but active, not dedifferentiated. Seventy-two hours, perfectly normal; detached spine dead. Eighty-four hours, dedifferentiated active sphere. Ninety-six hours, thick cyst.

## *2. Reversal of polarity in normal individuals*

In plate 5 are shown individuals of varying degrees of abnormality. Figures 27 and 28 are individuals nearly normal except for the spine. They had been in tap water without food, and were perfectly normal when isolated eighteen hours previously. Figures 29 and 30 were drawn eighteen and twenty-four hours respectively after isolation of perfectly normal individuals in tap water. The larger individual appears to be 'dominant.' Ciliation is unipolar except for cilia in the oral groove of the smaller end, which were beating backward. Figure 31 is a heteromorphic individual resulting from a normal individual after being isolated in weak solution of Horlick's malted milk. Figure 32 is the same individual drawn two hours later. The histories of the individuals shown in plate 5 were not followed further. They suffice however to show that reversal of polarity may arise without cutting and that different degrees of this tendency to form heteromorphic individuals occur.

## V. POLARITY, DOMINANCE AND RESISTANCE OF STRUCTURES TO DEDIFFERENTIATION

Whether dedifferentiation in *Bursaria* can lead to disappearance of polarity in the cell remains an open question, for although at the stage of the ciliated sphere which is shown in figure 7, there appears to be no visible anterior or posterior, neither in structure nor in swimming movements, nevertheless the protoplasm in the cyst has a polar axis (fig. 8).

The protoplasm of a normal posterior end may reverse its polarity and by dedifferentiation form a partial or complete anterior end (figs. 29, 30, 31, and 32). Similarly two anterior ends may arise simultaneously from the same dedifferentiated piece (figs. 21 a', 21 b). Heteropolarity can appear in ciliary action without any very marked morphological evidence of heteromorphic condition (fig. 22). Compare figures 29 and 30.

In every heteromorphic pair with members of unequal size, the smaller and weaker member sooner or later dedifferentiated and the whole or part of its substance became part of the stronger



and larger member (figs. 21 b, 23 a, and 26). In those cases where the members of the heteromorph were equal in size and degree of differentiation there seemed to be a balance, and if both of the individuals could take food and grow, the process led to the separation into two potentially equal individuals; for example figure 25. Other similar instances supporting these conclusions were observed but were not drawn. It is clear that in cases where there is inequality the larger piece has a tendency to exhibit a control or dominance over the smaller one, but as to its cause these observations tell us nothing (Child, '15, page 199). It is a peculiar fact that among all the heteromorphic individuals observed not a single one had the anterior ends pointing toward each other; that is to say no 'heteromorphic tails' appeared.

Another curious case illustrating how dedifferentiation may proceed when initiated by conditions slightly different from those described above, is the following. Figures 33 to 38 inclusive, are freehand drawings made as carefully as possible of six different conditions of an originally normal individual picked out from a culture which had been fed with grains of boiled yolk of hen's egg. When first observed (fig. 33), it was in the act of division but a string of yolk grains surrounded by a bridge of protoplasm prevented separation of the sister cells. This bridge of protoplasm remained as a connection between the individuals. Figure 34 shows the two individuals six and one-half hours later. Differentiation has proceeded normally in spite of the protoplasmic connection. After twelve hours the protoplasmic bridge widened and parts of the endoplasm of individual *B* were seen to flow into *A* by way of the connecting bridge of protoplasm. Individual *A* gradually became larger while *B* became smaller. This process continued until at twenty-nine hours after isolation *B* had passed entirely into *A* as shown by the fact that two distinct sets of quite fully differentiated active membranelles were present, and *A* had now become much larger (fig. 35). Increase in size was partly due to assimilation of the ingested yolk grains. For about ten hours between the stages shown in figures 34 and 35 the organism was not under observation, so

that the details of part of this process were not observed. Figure 34 is a dorsal view while figure 35 is a ventral view: from this it seems certain that the membranelles (*ma*) of figure 35 belong to individual *A* while membranelles of individual *B* would be those indicated by *mb* in figure 35. That this conclusion is correct may be seen from figure 36, a ventral view drawn forty-nine hours after isolation. In this *ma* is seen to be the original band of membranelles belonging to individual *A*, while *mb* is the remainder of the membranelles of *B*. Figures 37 and 38, drawn at seventy-two and eighty hours respectively show that the last traces of the membranelles of individual *B* were finally absorbed. Only the large macronucleus remained to indicate that this now apparently normal organism had once been made up of two normal individuals. The remarkable resistance of the differentiated gullet of individual *B* to absorption when compared to the ordinary process of digestion in this organism is noteworthy (Lund '14). It seems that under certain conditions active differentiated structures are relatively resistant to dedifferentiation.

#### VI. DEDIFFERENTIATION IN OTHER ORGANISMS

No attempt to review or abstract from the literature the facts relating to dedifferentiation in metazoa will be made. This has been done in an excellent summary and review by Schultz ('08). Schultz formulated the observations on metazoa in terms of the hypothesis that all phenomena of form regulation are somehow related to and a result of the reversal of differentiation or dedifferentiation. In other words regeneration is a result of the differentiation of dedifferentiated cells (See also Child, '15).

Many facts derived from careful studies on tissues, organs and whole organisms unmistakably point to this conclusion, and to the conclusion that one type of differentiated cell may transform into another type of cell having a different form and function. But as Schultz ('08) admits and as H. V. Wilson ('11) states, to prove it would necessitate following one and the same cell or its equivalent during the transformation. In the sponges, where H. V. Wilson ('11) has shown that a dissociation of the cells leads to partial or complete dedifferentiation with subsequent

rearrangement of the cells to form the beginnings of normal sponge structure, it has not yet been proven that all the dedifferentiated cells are equivalent in their potentialities and functions and actually can assume any of the rôles played by the different types of sponge cell.

In view of this status of the question the facts described for *Bursaria* may have an added interest in so far as they fulfil the necessary conditions for experimental proof that cell differentiation is in this case a reversible process.<sup>3</sup>

All that is implied in the expression reversibility of differentiation is that the parts or materials of a differentiated system when separated, somehow either reform a structure which is made up fundamentally of the same or duplicate elementary parts, but not necessarily having the same spacial relations to one another which they had in the original system, or are reformed by the same steps but in the reverse order. What evidence can

<sup>3</sup> It is important for what precedes and follows to make clear the meaning of the term reversibility. Let us represent a specific configuration of material parts by  $abcde---$ . A specific kind of protein molecule might be used as an illustration. The elementary parts represented by  $a, b, c, d, e$  would be the different amino-acids of which the specific molecule was formed.

We will assume that the reaction  $abcde---, \rightarrow a, b, c, d, e---$ , represents a dedifferentiation of the system  $abcde---$ , similar to the breaking down of a specific protein molecule into its constituent amino-acid groups. The reverse process,  $a, b, c, d, e, --- \rightarrow abcde---$ , would represent differentiation, a process of reformation of the system or synthesis. If now the reaction  $abcde---, \rightarrow a, b, c, d, e---$ , represented only the beginning and end results of a process which as a matter of fact took place in steps having a definite sequence (consecutive reactions), perfect reversibility would be represented by a reversed sequence of these steps or consecutive reactions. On the other hand if  $a, b, c, d, e, ---$ , united to form  $abcde---$ , but the sequence of the steps in this synthesis were not the reverse of the sequence of the steps for  $abcde---, \rightarrow a, b, c, d, e, ---$ , then the process would not be strictly reversible so far as the method of synthesis was concerned but would be considered reversible in so far as the end results were the same. Furthermore if  $abcde---$ , separated to form  $a, b, c, d, e$ , and these elements reunited to form  $debca---$ , then the process would be considered still less perfect in its reversibility. However, the process could be said to be reversible in so far as it led to the separation and reformation of complexes which had the same elementary percentage composition. Chemically speaking  $abcde---$ , and  $debca---$ , would be isomers. All these degrees of reversibility are exemplified in various syntheses by enzymes. See Euler, General Chemistry of the Enzymes, page 261.

we find in support of such an interpretation of the process of dedifferentiation and differentiation?

#### VII. RÔLE OF REVERSIBILITY IN CELL PROCESSES

Reversibility of many processes in living matter is one of the fundamental dynamic characteristics of organisms. A physical-chemical system which is reversible is a self regulatory system at least in so far as its equilibrium conditions are concerned. Living organisms make use of a large number of such self equilibrating systems, for example the mechanism for maintaining the acid-base equilibrium in the body; many chemical mechanisms concerned with translocation of fats, carbohydrates and proteins in plants and animals under different conditions; the reversible character of the permeability of plasma membranes. The results of Loeb ('14) indicate that the processes antecedent to cleavage in artificial parthenogenesis have a reversible character. Many other instances of reversibility might be mentioned. Evidence of visibly reversible gelation and liquefaction transformations in living protoplasmic structures of cells has been given by Kite ('13) and recently by Chambers ('15) and ('17). They seem to be very similar in nature to the processes described for *Bursaria*, and suggest very strongly that differential gelation and liquefaction processes play a large rôle in cell morphogenesis.

To what known biological or chemical processes can we look for an explanation of such gelation and liquefaction processes in living cells? I believe that many intracellular chemical processes of which we have at present some definite knowledge will help to throw light on the mechanism of dedifferentiation. I have in mind particularly the familiar phenomenon of autolysis. Many different endoenzymes, in themselves perhaps the expression of surface effects (Bayliss, '14) occur in protoplasm, whose actions are inhibited and controlled under normal conditions of metabolic equilibrium. Autolysis leading to a disintegration of complex proteins (dedifferentiation?) of the cell takes place in practically all cells when the normal income of the cells is withheld. For example in the yeast cell, so long as food is available there is little evidence of self digestion. But

if food is withheld, a very active protein-splitting enzyme is soon developed, whose activity leads to disintegration of the protoplasm and proteins of the cell with the appearance of amino acids and other relatively simple substances.

Protein decomposition and synthesis is a reversible process in the sense in which the term reversibility is used in this paper (defined above). Proof that living cells normally contain or can develop a mechanism for a reversible process of cleavage and synthesis of their own proteins is practically complete (Levene, '05).

From this viewpoint, dedifferentiation, such as described for *Bursaria* and such as may be seen to a less striking degree in many other ciliates should not be considered an exceptional phenomenon, but rather one which occurs to a wide extent among different types of cells.

That dedifferentiation in *Bursaria* is distinct from the process of breakdown and utilization of the cell substances for energy requirements was shown by the fact that large individuals measuring over 500 microns in length, when placed in tap water and starved for several days decreased in size until in many observed cases the length of the individual was less than 90 microns. Many of these starved individuals had fully differentiated and normal gullets. In the starved individuals there is decrease in size of the gullet but proportions are maintained. Dedifferentiation may or may not occur during starvation. Often differentiation of a dedifferentiated individual was seen to occur after two or even three days of starvation in tap water when the size of the individual was much decreased. These facts show that dedifferentiation in *Bursaria* and probably in many other kinds of cells is a rather distinct type of process, and that we are not justified in considering it as identical with the ordinary extensive autolytic changes in cells *in vitro*, or with katabolic processes which supply the energy requirements of the cell, but rather as a related type of breakdown of colloidal complexes; this breakdown being inhibited and controlled in a similar way to the inhibition and control of the action of endoproteolytic enzymes. According to experiments by Levene ('05) and more recently by



Bradley ('15) on the autolysis of liver cells, such a controlling factor seems to be the hydrogen ion concentration.

I am unable to conceive how from the standpoint of physical or chemical phenomena a 'making over,' 'regulation,' 'reorganization,' 'reconstitution,' 'rejuvenation' of a differentiated part of a cell can be accomplished so as to supply missing parts of a different structure unless the differentiated parts which remain are first broken down or simplified, at least to some extent, and then rebuilt to form the normal structure. Furthermore evidence points to the conclusion that mechanisms for such breakdown of cell structure are of wide occurrence in many cells.<sup>4</sup>

#### VIII. RELATION OF MORPHOGENETIC PROCESSES TO HEREDITARY CONSTITUTION OF BURSARIA

If we should assume that the determiners for the structures in Bursaria were 'located' in the nuclear mechanism we would have to consider the parts played by the macronucleus and the micronuclei. So far as the macronucleus is concerned we have seen that regulation leads to the same result whether a big or a small piece of the macronucleus is present; evidently any part of the macronucleus is equivalent to every other part so far as regeneration of the missing structures is concerned. If the macronucleus contains the determiners for the cell characters evidently there must be many like determiners for the same character, distributed throughout the macronucleus. It is impossible to apply a similar analysis to the micronuclei, for in Bursaria there are from 9 to 15 or more micronuclei and some of these are probably always included in a piece. So far as I am aware Calkins ('11, page 104) is the only investigator who has

<sup>4</sup> To make the idea clearer by using a crude illustration: if half of a masonry house having a definite architectural design (differentiation), was removed, and it was required to make a house out of the remaining half, of the same architectural design as the original, then the simplest and only possible way would be to tear it down and rebuild a smaller one out of the stones that remained in the part of the old house. The process of rebuilding (redifferentiation) would be conditioned by the isolation of the stones of the remaining structure (dedifferentiation) which now would be available for recombination to form a new structure of the same architectural design but half the size of the original one.

reported that a piece of a ciliate, in this case *Urorychia*, containing a piece of the macronucleus but no micronucleus is capable of regenerating into a normal individual. This point is of interest and ought to be investigated further.

The fact to be noted in this connection is that whenever dedifferentiation occurs it is limited in its extent and does not lead to any visible disintegration or dedifferentiation of nuclear substance. There is always a differentiated residuum which might be considered to have a relatively stable germinal differentiation and doubtless has, for redifferentiation of a dedifferentiated piece leads to formation of a definite hereditary pattern. From this it is evident that dedifferentiation is limited to relatively superficial and transient cell structures.

In connection with the fact that dedifferentiation is limited in its extent to superficial (somatic) structures and does not affect germinal or nuclear structures, and the theory proposed above that the dedifferentiation process is closely related to phenomena of autolysis, it seems to me significant to note that nuclei of tissues are much more resistant to autolysis than the cytoplasm. A residuum remains after prolonged autolysis of tissues, which consists largely of nuclei and nuclear debris. This is chemical evidence indicating that nuclear structures are chemically speaking relatively stable, and hence on the above interpretation we should expect that dedifferentiation would visibly involve only cytoplasmic structures, which it does. I can verify this point from observations on various protozoa; for example *Colpidium* and *Paramecium* which were ingested by *Bursaria*. The macronucleus persists in a recognizable condition much longer than the cytoplasmic structures of the food material in the digestive vacuole.

#### IX. SUMMARY

1. Dedifferentiation of the gullet in the ciliate *Bursaria* always occurs before division, encystment, and regeneration of lost parts of the cell. It also occurs spontaneously and is not always followed by division, encystment or regeneration.

2. Encystment follows complete disappearance of the anlage of the gullet, but polarity of the cell is probably not lost. During cell division the anlage of the gullet remains and from it a new gullet is formed by differentiation. The extent of cytoplasmic dedifferentiation varies with the degree of mechanical injury and size of the lost part.

3. Heteromorphic cells may arise from either normal individuals, anterior, posterior, right, or left halves. In every case differentiation took place in a cell or part of a cell which was first dedifferentiated.

4. Reversal of polarity of one of the members of a heteromorph may occur as a result of dedifferentiation of that member. Reversal of polarity and dedifferentiation in all observed heteromorphs took place in the smaller and weaker member. Heteropolarity may occur with very little other evidence of its existence than the direction of beat of the cilia.

5. The morphogenetic processes in *Bursaria* and probably in many other cells are reversible (defined page 13) in their nature. One phase of this reversible system is represented by differentiation, the other by dedifferentiation.

6. Evidence for the existence of a specific type of physical-chemical mechanism for dedifferentiation similar in its action to the mechanism of autolysis, is given in the text.

7. The reversible morphogenetic processes described for *Bursaria* concern only changes in relatively unstable, secondary structures in the cell and do not visibly affect the more stable nuclear mechanism.

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## PLATE 1

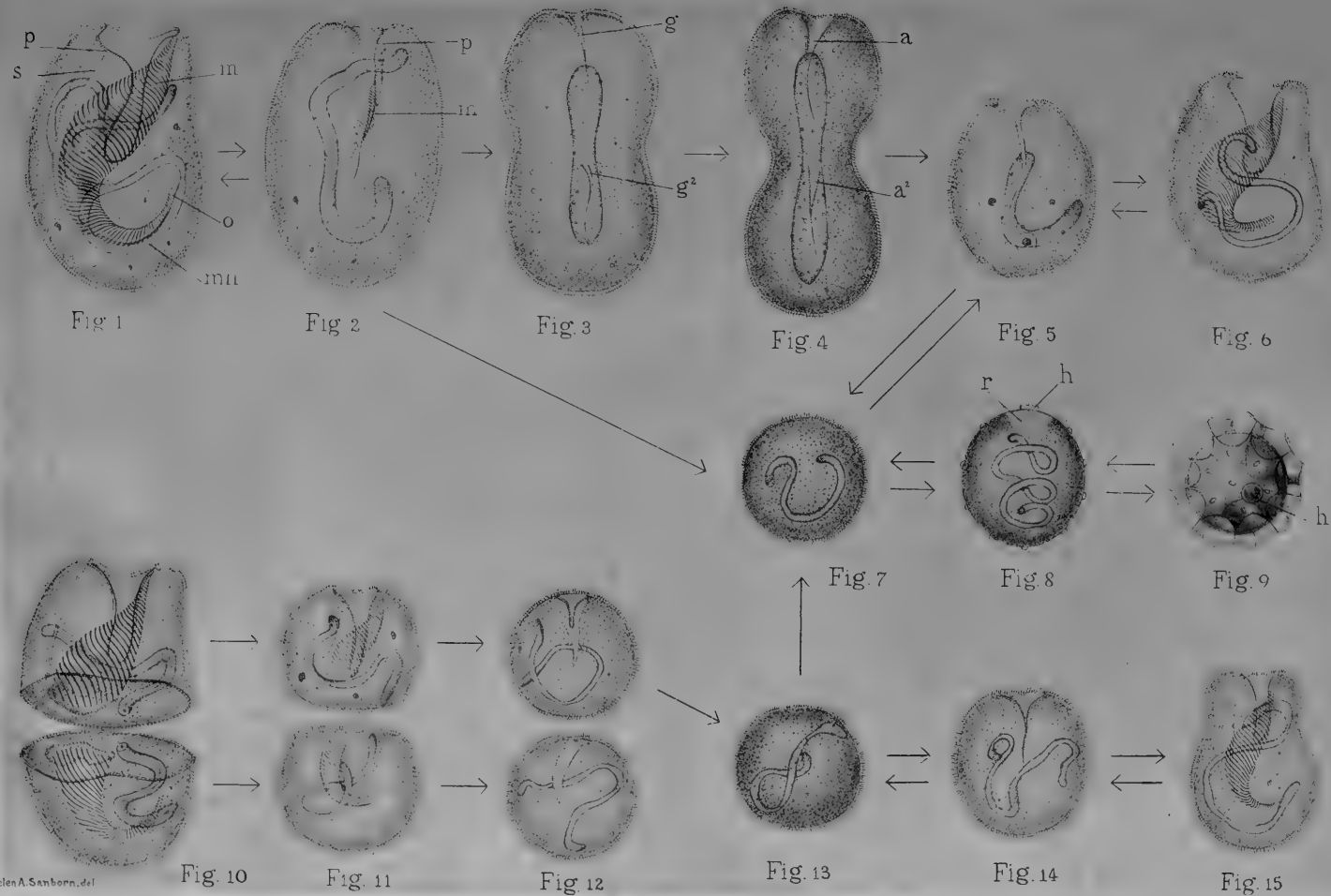
### EXPLANATION OF FIGURES

1 to 6 Stages in normal division. *p.*, peristome; *s.*, septum; *m.*, membranelles; *g.* and *g*<sup>2</sup>, remains of the gullet after dedifferentiation. The gullet is to be considered as made up of the invagination containing the membranelles, septum, etc., in figure 1. *g.*, and *g*<sup>2</sup>, are the same as *a.*, and *a*<sup>2</sup>, the anlagen of the new gullet.

7, 8 and 9 Encystment. *h.*, hilum; *r.*, clear body of protoplasm. The upper arrows between figures 9, 8 and 7 refer to the reversal of the morphogentic processes which take place in the protoplasm and not the cyst.

10 to 15 Stages in the process of regeneration. Figure 10 is partly schematized for the sake of clearness, by showing the cut surface as it would appear if it did not close. The arrows show the possible sequence in the processes of dedifferentiation and differentiation.





## PLATE 2

### EXPLANATION OF FIGURES

16 Cross section through the middle of one of the dedifferentiated sister cells at division shown in figure 4. *a.*, anlage or remains of the gullet after dedifferentiation.

17 Same as figure 16. but at beginning of differentiation of gullet as shown in figure 5.

18 Cross section through middle of fully differentiated cell. From same level as figures 16 and 17. *m.*, membranelles; *s.*, septum; *g.*, gullet.

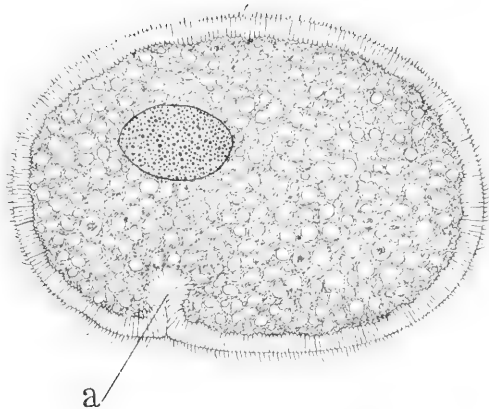


Fig. 16

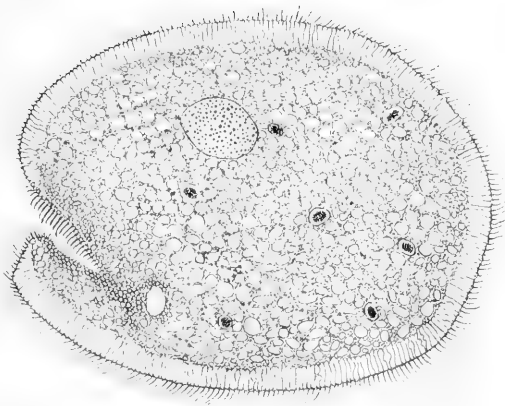


Fig. 17

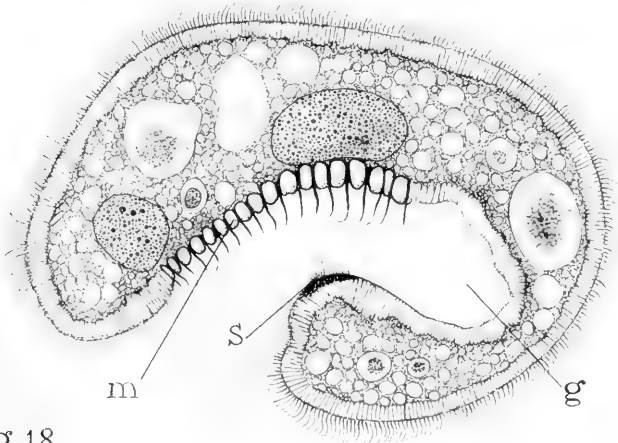


Fig. 18

Helen A. Sanborn, del.

### PLATE 3

#### EXPLANATION OF FIGURES

19 Longitudinal sagittal section of a fully differentiated individual. *p.*, peristome; *m.*, membranelles; *g.*, gullet; *f.*, food vacuole; *mn.*, macronucleus; *v.*, ventral; *d.*, dorsal.

20 Horizontal longitudinal section of a spontaneously dedifferentiated normal individual. Dedifferentiation more complete than at the stage shown in figure 2. *a.*, anlage of the gullet; *mn.*, macronucleus. Miconuclei not shown.

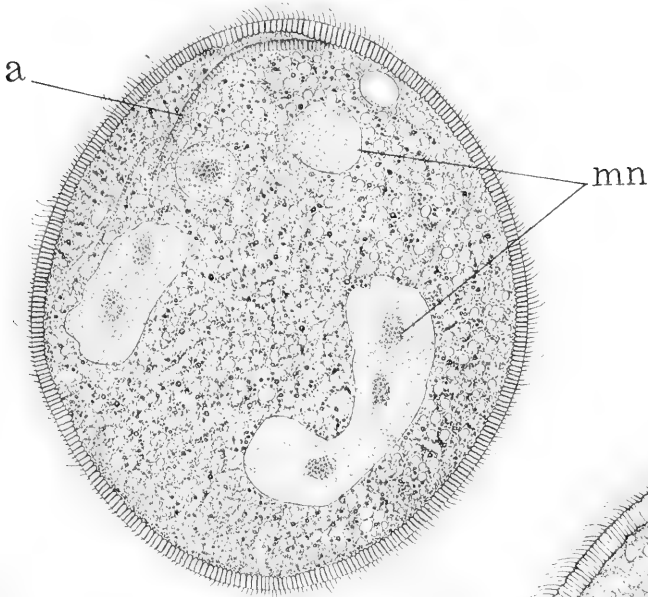


Fig. 20

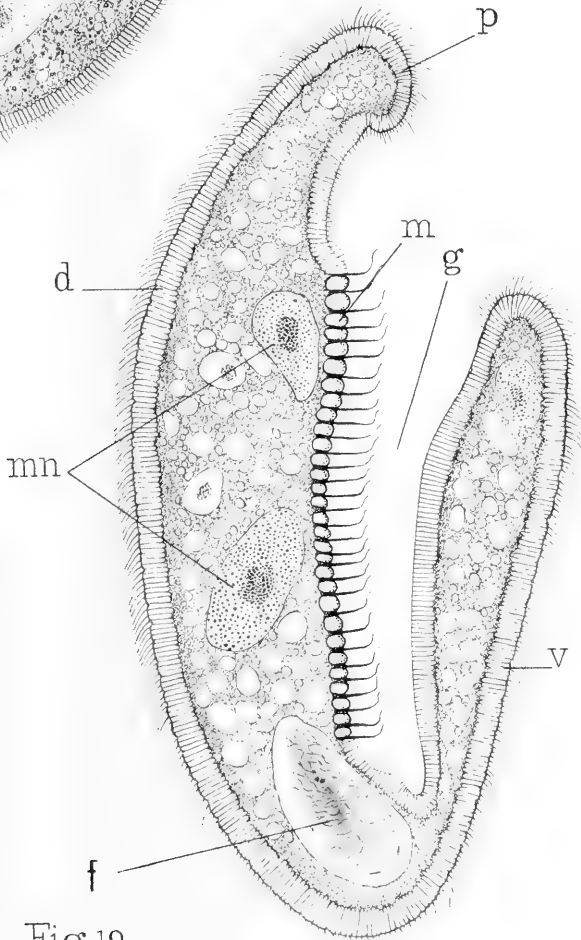


Fig. 19

## PLATE 4

### EXPLANATION OF FIGURES

All figures are of heteromorphic individuals derived from cut halves of normal individuals. See page 8 of text for full description and history.





Fig 25

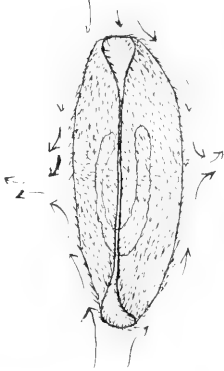


Fig 23b



Fig 26

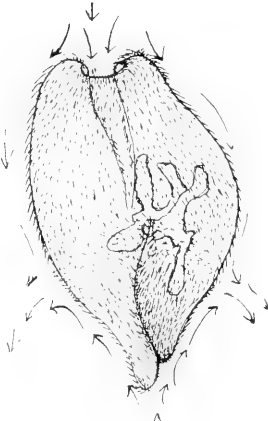


Fig. 23a

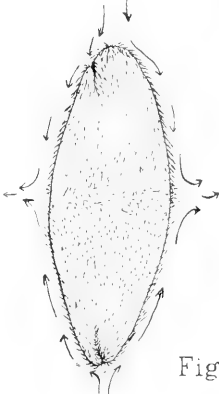


Fig 22

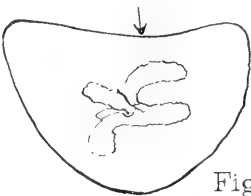


Fig. 21a



Fig 24

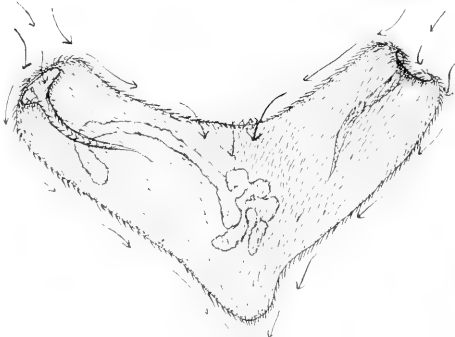


Fig 21a'

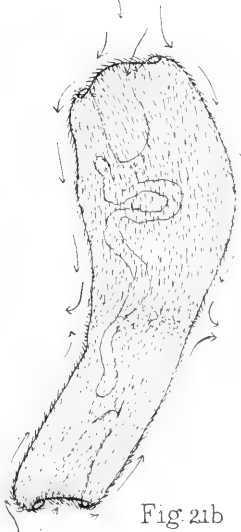
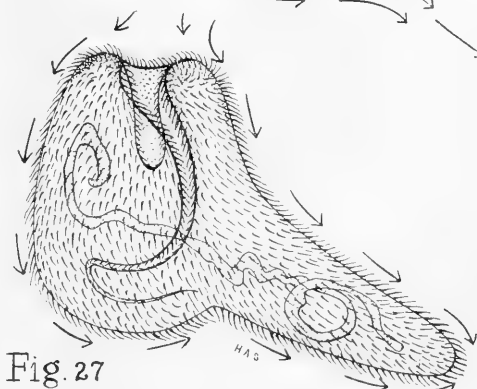
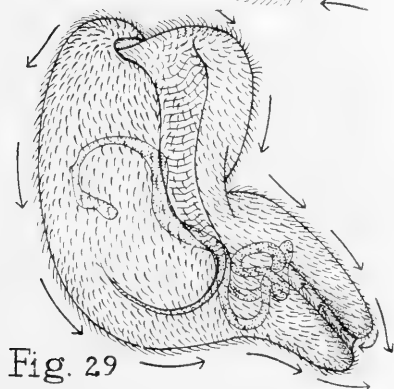
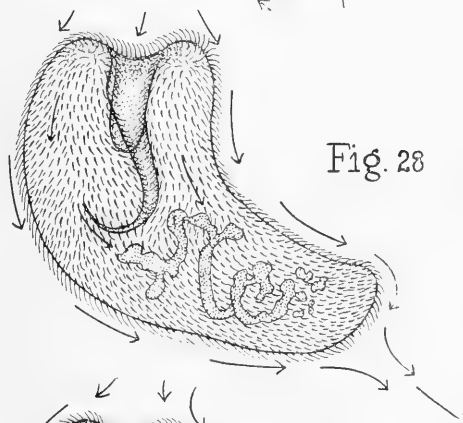
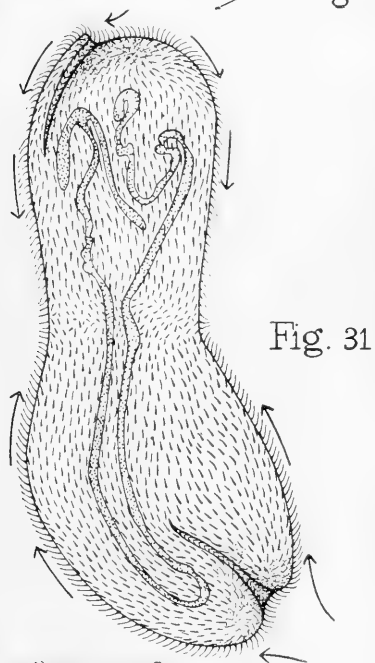
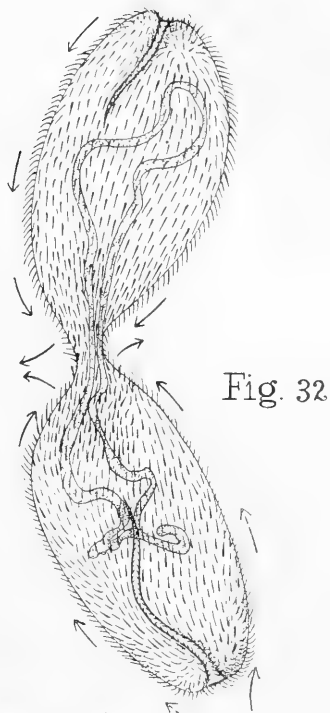
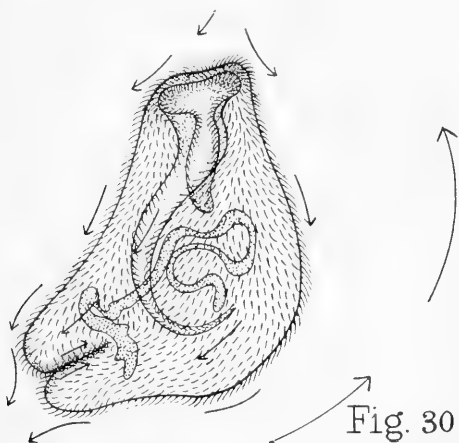


Fig 21b

## PLATE 5

### EXPLANATION OF FIGURES

Abnormal and heteromorphic individuals developed from normal individuals put into tap water. See page 9 of text for full description and history.



## PLATE 6

### EXPLANATION OF FIGURES

33 to 38 Free hand drawings of stages in the process of absorption of individual *B* by individual *A* of figures 33 and 34. *b.*, protoplasmic bridge surrounding the row of yolk grains and connecting the sister cells *A* and *B*; *ma.*, membranelles of individual *A*; *mb.*, membranelles of individual *B*; arrows indicate the direction of ciliary currents. Full description and history on page 11 in text.

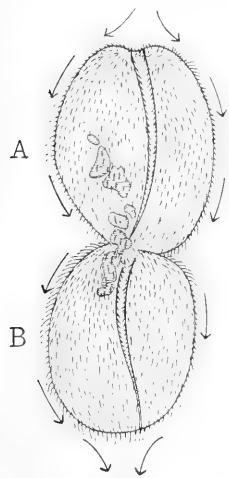


Fig 33

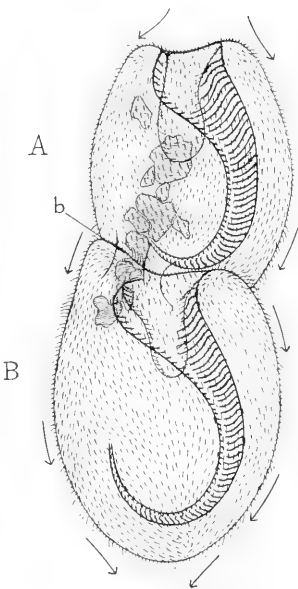


Fig 34

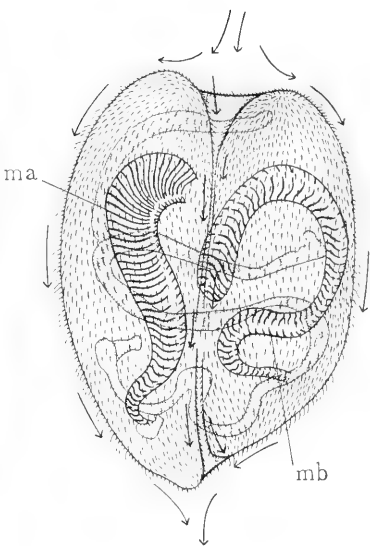
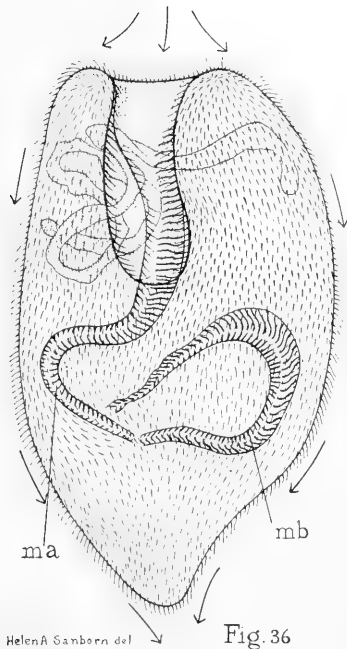


Fig 35



Helena Sanborn del

Fig 36

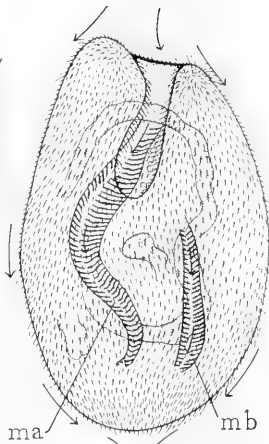


Fig 37

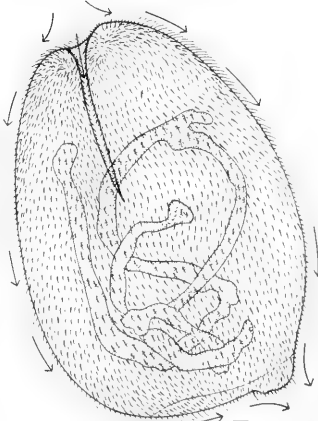


Fig 38



## EXPERIMENTAL INDUCTION OF ENDOMIXIS IN PARAMAECIUM AURELIA

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THREE PLATES

Variations from the usual number of both macro- and micro-nuclei in Infusoria have been described by several investigators, Hertwig ('88-'89), Calkins ('04), Popoff ('07, '09), etc. Popoff ('09), Sun ('12) and Kasanzeff ('01) have shown that these nuclear changes can be induced by various means (starvation, temperature and chemical treatment). Hertwig (l.c.), Popoff (l.c.) and Sun (l.c.) have pointed out the similarity between these stages and those occurring in conjugation, and the two former authors have compared them to parthenogenesis in the metazoan egg, which can here, as there, be artificially induced (Popoff, '09). This interpretation has been amplified by Woodruff and Erdmann ('14, '16) and accepted by Calkins ('15), although the former writers have proposed the term 'endomixis' instead of 'parthenogenesis' for this phenomenon.

According to these latter writers nuclear re-organization serves to rejuvenate a depressed race. This interpretation seemingly contradicts the work of Jennings ('13), who has shown that conjugation does not result in rejuvenation, but is to be interpreted as a means of producing bi-parental inheritance and variability in the offspring.<sup>1</sup>

Woodruff and Erdmann (l.c.) have shown further that the occurrence of endomixis does not necessarily accompany a depression period (see line III, p. 480). Regarding this irregularity the authors state that it "is an exception for which the data afford no adequate explanation." Their graphs (text figs.

<sup>1</sup> See also Mast ('16) in this connection.

16 and 17, pp. 479 and 480) show moreover that periods of low division rate may ensue without the occurrence of the rejuvenating process. I do not here refer of course to the low division rate period in line VI which was induced by bacteria, but rather to that just preceding the first occurrence of the process in line VI (text fig. 16) and to the two low periods in line III (text fig. 17) between the first and second occurrence of the process, and that just preceding its last occurrence in this line. They also show that the process may commence, but not continue to its completion (p. 458). The culture method employed by these authors renders their conclusions uncertain. The frequent changes of culture fluid (i.e., after every two divisions) to which they subjected their *Paramaecia* might have enabled these to survive the depression periods through which they passed, and enter upon new periods of activity, and at the same time to recover from the nuclear disturbance which they simultaneously experienced. Calkins ('02 et seq.) was able several times to rehabilitate his *Paramaecia* by subjecting them to changes of culture media, and it is a well known experience that *Paramaecia* will develop rapidly when transferred from an old to a fresh culture. Thus it is not unreasonable to ask whether the transfer to a fresh culture was not what enabled specimens to survive their periods of depression and enter upon subsequent ones of active reproduction. In order to prove their point it would be necessary to carry the *Paramaecia* through the reorganization process in the same culture without the frequent culture changes made by them.

While examining a culture of *Paramaecium aurelia* for class work I found the multinucleate condition occurring commonly, and since then have found it frequently in cultures derived from the original material as well as from other sources, and in *P. caudatum* as well as *aurelia*.

In order to examine further the significance of multinuclearity, and to test the possibility of its experimental induction, I have performed a series of experiments which I shall here describe, and which I have designed to answer the following questions:

- 1) Can endomixis be experimentally induced?



2) Is endomixis an evidence of depression or a means of rejuvenescence?

1. Can endomixis be experimentally induced? In order to gain further evidence on this point I have performed a series of experiments using the daily isolation method of culture, and employing cells of the same ancestry, on the one hand for the experiment, and on the other for the control.

Popoff's work tends to show that various end products of metabolism, such as  $\text{CO}_2$ ,  $\text{NH}_3$  and even  $\text{CO}(\text{NH}_2)_2$  may induce multinuclearity. Woodruff ('11) has shown that excreta of *Paramecium* are depressing agents, lowering the division rate, and Woodruff and Erdmann (l.c., p. 485) have briefly stated that staleness of culture medium hastened the onset of endomixis in *P. aurelia*. In order to ascertain whether such depressing agents, namely excreta, serve to induce multinuclearity or at any rate to increase the frequency of its occurrence, I have carried several lines of *Paramecium* in culture fluid containing large amounts of excreta for several weeks, and then have changed the culture to fresh medium in which excreta were present in small quantities. I have further carried sister lines in parallel cultures, one of which contained more, and the other less excreta. The amounts of excreta employed varied in the different experiments, but in no case have I attempted to measure the exact amount used. In some experiments I have merely left the animal on the same slide, removing as much as possible of the old, and adding fresh culture daily, and occasionally transferring it to a fresh slide, when the old one became too foul with Bacteria. In another I have used an old culture in which *Paramecia* and other Infusoria and Bacteria had lived for many months and from which they had finally almost or entirely disappeared. The fresh culture medium employed except as otherwise noted was a 0.025 per cent beef extract solution in ordinary distilled water.

It is true that the excreta employed in these experiments were not derived from *Paramecium* alone. Bacterial products were present in all, and excreta of several other species of Infusoria in some of the experiments. The point is immaterial, however, as what I have endeavored to determine is whether

depressing agents, regardless of their character, will affect the nuclear condition of *Paramaecium*.

In these experiments the animals were kept at room temperature, varying from 13° to 25°C.<sup>2</sup> For the most part the room was partly lighted during the day from a west window and at night was dark. The nuclear condition at each isolation with few exceptions was determined by staining with methyl green. In a few cases permanent mounts stained in haematoxylin were made. This was not done daily, however, because of the labor involved, and because I was only interested in the gross condition of the macronucleus, whether single or multiple. The details of the nuclear changes in endomixis have been worked out by Woodruff and Erdmann and do not concern us here.

The frequency of endomixis I have determined by the number of generations between two of them, rather than by the time interval, as the former indicates better than the latter the amount of growth between the periods.

*Experiment 1 (figs. I and Ia)*

In this experiment line I was carried for one-hundred and eighty (two) generations<sup>3</sup> on the same slide, with occasional changes to a fresh slide. The influence of a change of slide with consequent nearly complete change of culture was usually shown in the marked increase of division rate of the animals. Occasionally, however, such an increase appeared when no apparent external cause was responsible. Such increases moreover were not invariably associated with endomixis. After one hundred forty-seven (eight) generations line Ia was branched off and carried with fresh slide changes daily from 5/8 to 6/19. The average daily division rate, during the first part of this experiment, when the slide was changed only occasionally, was 1.4; while during the latter

<sup>2</sup> In experiment 2, line II (2/22-4/15, 4/17-5/2) the slide was carried in a thermostat, ranging between 19° and 22° C. and in experiments 2, 4, 5, 6, and 13, lines IIa, IV, V, VI and XIII. (5/23-6/19) at 20°-23°C. as a check on those conducted at room temperature, but without noting any difference in the results. The range of 12°C. to which the animals were subjected at room temperature more nearly simulates natural conditions, than does that of only 2°-3° maintained in a thermostat. The extremes of 13° and 25° in room temperature were experienced but rarely and for short intervals. The thermostat lines necessarily experienced a slight change of temperature daily during periods of examination.

<sup>3</sup> Uncertainty regarding the counts in two cases renders the total number of generations uncertain to the extent of two. The point is immaterial however.

part, when daily changes were made, the rate was 2, showing a marked increase. During the former period endomixis occurred on an average of once every twenty-five generations, while during the second part its occurrence was reduced to once every ninety-one (two) generations,<sup>4</sup> showing the marked influence of purity of culture medium in reducing the frequency of endomixis.<sup>5</sup>

#### *Experiment 2 (figs. II and IIa)*

In this experiment line II was branched off from I on 2/8 and carried through one hundred and six generations with only occasional changes to fresh slides, two or three drops of fresh culture however, being added daily. From 4/21 to 5/6 a 0.5 per cent solution of urea in distilled water was used as a culture medium in an effort to induce endomixis. This failing, the line was returned to beef extract on the latter date. On 5/9 line IIa was branched off from II and carried until 6/19 with fresh slide changes daily. The details of the experiment are shown in the accompanying graphs, which show that in line II endomixis occurred on the average of once in every thirty-five generations<sup>6</sup> while in line IIa it occurred once in seventy-eight generations, an increase of forty-three generations.

It is possible that the change to 0.5 per cent urea solution followed by a re-transfer to beef extract may have induced the long interval of 65 generations between the endomixis of 3/5 and 5/13 in line II. This suggestion is borne out by the fact that in line IV, which was branched off from II on 4/13 and carried without change of slide until 5/12, endomixis occurred on 5/1 forty-five generations after the last precedent one in line II, which appeared on 3/5. It is also supported by the results of Calkins ('02-'04) who showed that various stimuli (change of food, temperature, shaking and chemicals) tend to increase the division rate.

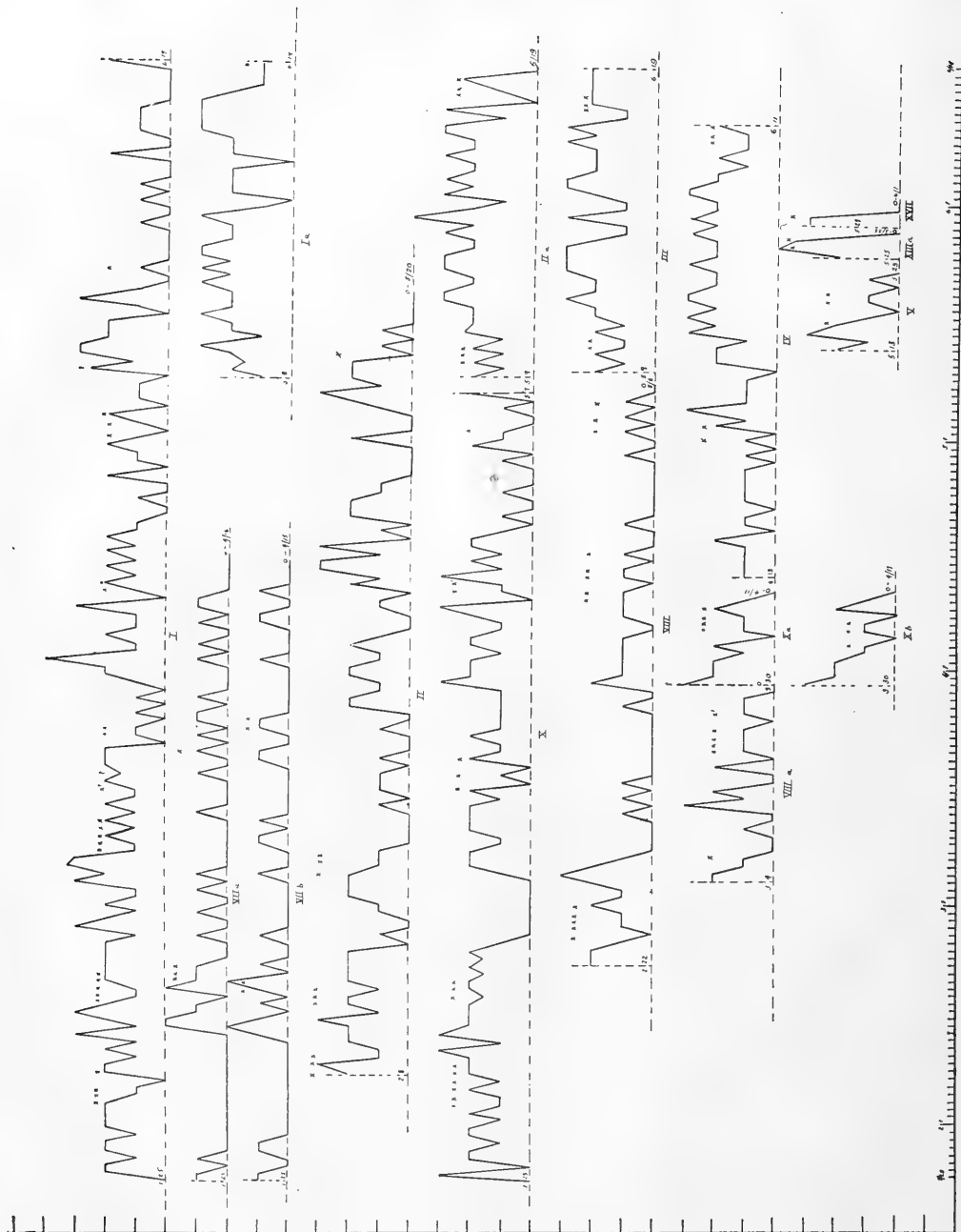
#### *Experiment 3 (fig. III)*

On 5/9 line III was branched off from II and carried with daily slide changes until 6/19, endomixis occurring on 5/13 and 6/13, with an intervening period of sixty-four generations, an increase of twenty-nine over thirty-five, the average period in II, the parent line.

<sup>4</sup> Counting from the last endomixis in line I before Ia was branched off from the latter.

<sup>5</sup> My notes show an endomixis in line I on 3/16. This is in all probability an error because of its close proximity to those on 3/8 and 3/23, and because the notes give no record of multinuclearity on either 3/15 or 3/17. I have accordingly rejected it in computing the average period of 21 generations between endomixes in this line. If it were counted, however, it would reduce the average to less than 21 and make the contrast even greater between the average period in line I and the period in Ia.

<sup>6</sup> Counting from the appearance of the last endomixis in line I from which II was branched off.



*Experiment 4 (fig. IV)*

In this experiment line IV was branched off from II on 4/13 and carried for twenty-six generations with infrequent changes to fresh slides. After 5/9 slide changes were made daily until the termination of the experiment on 6/11. Between 3/5, the date of appearance of the last endomixis in II, the parent line, and 5/1 when the first endomixis occurred in line IV, there was a period of forty-five generations; while from 5/1 until 6/9 when the next endomixis ensued in line IV, there was a period of seventy-six generations, an increase of thirty-one.

*Experiment 5 (fig. V)*

On 5/13 line V was branched off from IV and carried without change of slide until 5/23, an endomixis ensuing on 5/17, twenty-four generations after the last endomixis in line IV from which this line was branched. Here we see that instead of a period of seventy-six generations between endomixis, as occurred between 5/1 and 6/9 in line IV, the sister line of V, a period of but twenty-four generations occurred.

*Experiment 6 (figs. VI and VIa)*

On 4/15 line VI was branched off from line II and carried with occasional slide changes in a weak solution of  $\text{NH}_3$  in beef extract<sup>7</sup> until 4/29 in an effort to induce endomixis, when it was transferred to beef extract on a fresh slide, and carried to 5/6 with one change of slide, after which it was changed daily until 6/16. Endomixis occurred on 4/23, forty-eight generations after its last occurrence in line II, and one on 6/14, 102 generations later. On 5/9 line VIa was branched off from line VI and carried with infrequent slide changes until 6/17. An endomixis ensued on 5/15, 19 generations after the last in line VI. Here we find a period of but 19 generations between endomixis in a line carried with but few slide changes, while in one with daily changes<sup>8</sup> one hundred and two generations intervened.

*Experiment 7 (figs. VIIa and b)*

In this experiment two lines VIIa and b were carried with infrequent slide changes for thirty and twenty-six generations respectively in an old culture in which Paramaecia and other Infusoria had been living for several months, the average interval between endomixis for

<sup>7</sup> Exact strength not recorded.

<sup>8</sup> Except the first eleven generations 4/23 to 5/6.

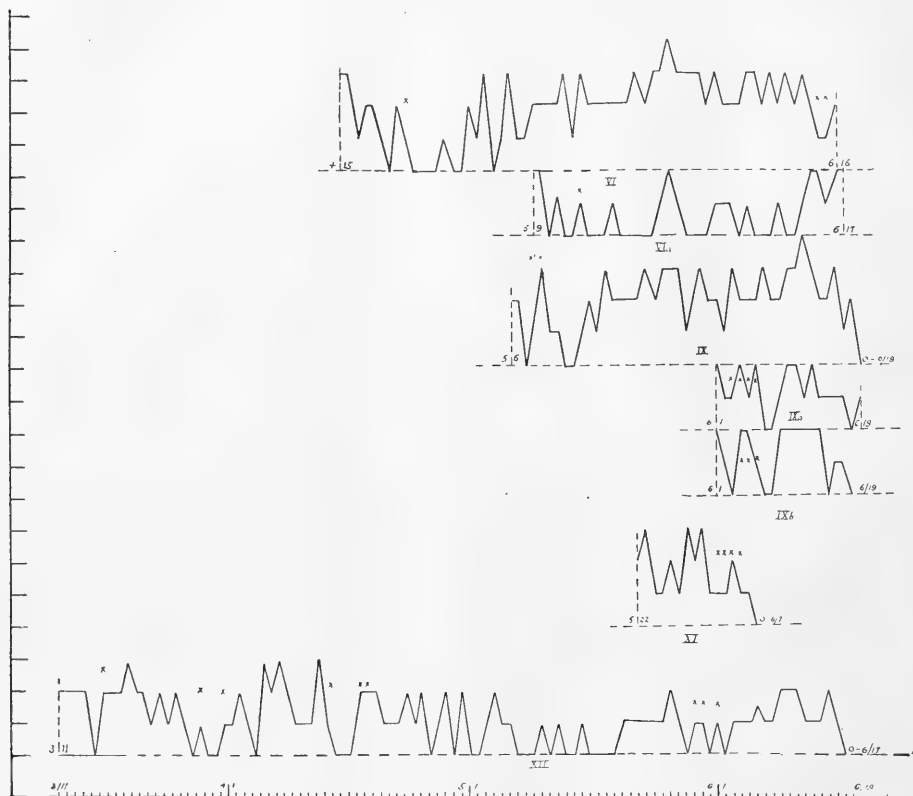
## DESCRIPTION OF FIGURES

The graphs show the daily division rate, vertical divisions representing one division and horizontal divisions one day. 'x' signifies the occurrence of endomixis. 'x?' indicates uncertainty regarding its occurrence and '?' uncertainty regarding the number of divisions.

the two lines being only ten. Further reference to this result will be made in the general discussion.

*Experiment 8 (figs. VIII and VIIIa)*

On 2/22 line VIII was branched off from line II and carried without change of slide in beef extract until 3/1. On this date the line was transferred to a fresh hay infusion on another slide, and carried,



with infrequent slide changes, in the same culture until its death on 5/8.

Several descendants of line VIII were sowed in the culture jar and a weak culture developed, which however died previous to 4/19, for what cause I cannot say. Endomixis occurred in line II on an average of once in thirty-five generations, while in line VIII it occurred once in eighteen and one-half generations, counting from the appearance of the first endomixis on 2/25. It is noteworthy that between this and the next endomixis on 4/10 a period of thirty generations ensued,

which was only slightly less than the average in line II, while between the latter endomixis and the next one in line VIII (5/2) there were only seven generations.

On 3/4 a second line VIIIa was branched off from VIII and carried in the same culture without change of slide until its death on 3/30. During this time it passed through twenty generations and two periods of endomixis (3/7 and 3/21) with intervening periods of only thirteen and ten generations between the appearance of the last endomixis in line VIII and the first in line VIIIa, and between the two occurrences of endomixis in the latter line, or an average of eleven and one-half. Why frequency of endomixis should have been increased by the use of this culture is not clear. Approximate bacterial counts showed large numbers of bacteria present, so that the cause was probably not lack of food. The *Paramaecia* growing in the culture itself and those on the culture slides were rather small and did not show the prominent food vacuoles usually present in healthy animals. It is more likely that poisons of some sort inhibited active growth and prevented the animals from using the food present. Whatever the explanation it is evident that conditions inhibiting growth induced endomixis. It is also worthy of note that initially the culture did not have so pronounced an effect as finally, the frequency of endomixis being increased in line VIII from thirty at the beginning to seven generations at the end of the experiment, and in line VIIIa from thirteen at the beginning to ten at the end.

*Experiment 9 (figs. IX, IXa and b)*

On 5/6 line IX was branched off from line II and carried with occasional slide changes until 5/14 when it was transferred to a fresh slide and carried until 6/19 with daily slide changes. Endomixis occurred on 5/8<sup>9</sup> and none thereafter for eighty-four generations until the death of the line on 6/19. On 6/1 two lines (IXa and b) were branched off from IX and carried until 6/19 in an old culture, with endomixis ensuing on 6/3<sup>10</sup> forty-five generations after its last occurrence in line IX and only one generation after IXa and b were branched off from the latter.

Another line of experiment consisted in using distilled water as the experimental agent, while beef extract was used as the control. The distilled water used in these experiments was

<sup>9</sup> No division occurred on 5/8, while my notes are uncertain regarding the nuclear condition on 5/9. The form of the curve however, and the existence of multinuclearity on 5/10 are sufficient evidence for believing that endomixis occurred on 5/8.

<sup>10</sup> Since IXb he did not divide on 6/3 it is of course impossible to be certain that endomixis occurred in that line on that date. On 6/4 the macronucleus had reformed however, so that there can be no reasonable doubt of this fact.

kindly prepared for me by Dr. W. W. Hanford of the State Public Health Laboratory, and was ammonia free at the outset of the experiments, while at their close several weeks later it contained only a trace. It was applied in gradually increasing amounts, starting with mixtures varying from one drop of distilled water to one of beef extract solution, to one drop of the former to five of the latter, and increasing up to pure water.

*Experiment 10 (figs. X, Xa and b)*

In this experiment line X was carried in beef extract for one hundred and thirty-one generations from 1/25 to 5/7 with infrequent slide changes. On 3/30 two lines (Xa and b) were branched off from X and carried without change of slide until 4/11. The culture fluid used at the outset of the experiment was a 5:1 mixture of beef extract solution and distilled water. This was gradually diluted to pure distilled water on 4/11 when both lines died. Endomixis ensued on 4/4 in line Xb seven generations after its start, and in line Xa on 4/5<sup>11</sup> nine generations after its start; while in line X the next endomixis occurred on 4/10,<sup>12</sup> eighteen generations after Xa and b were branched off from it. It is interesting to note that both lines Xa and b died, Xa immediately and Xb very shortly, upon transfer to pure distilled water on 4/11. While the division rate was notably reduced prior to their death, they were nevertheless able to survive great dilution of the culture medium with H<sub>2</sub>O, but were not able to bear transfer from the greatly diluted medium to pure H<sub>2</sub>O itself.

*Experiment 11 (fig. XI)*

In this experiment line XI was branched off from line IV on 5/22 in a 1:1 mixture of beef extract solution and distilled water and carried gradually into pure distilled water on 6/3 with daily changes of slide. Endomixis occurred on 6/1 seventeen generations after the line was started, whereas in the parent line IV it did not occur until 6/9 thirty-eight generations after.

In the following experiments temperature was the experimental agent, the experimental lines being suddenly transferred

<sup>11</sup> Since there was no division of line Xa on 4/5 it is impossible to say whether endomixis occurred then or not until 4/6 when the first record of it was obtained. The cessation of division of 4/5, and the nuclear conditions on 4/6 are strong presumptive evidence of its occurrence on 4/5. The point is immaterial in any event.

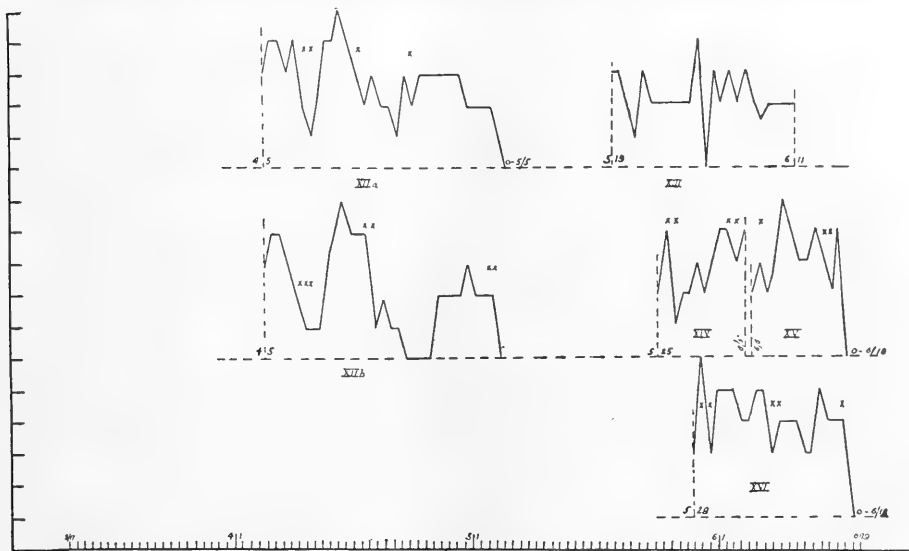
<sup>12</sup> My record regarding this endomixis is uncertain. In all probability one occurred at this time. But if it did not occur, then the influence of the distilled water in inducing endomixis in lines X a and b becomes more pronounced than if it did.



from room temperature at approximately  $20^{\circ}$  to a thermostat at  $29^{\circ}$  to  $30^{\circ}$ .

*Experiment 12* (figs. XII, XIIa and b)

In this experiment line XII was carried with infrequent slide changes from 3/11 until its death on 6/17. On 4/5 lines XIIa and b were branched off and carried with infrequent changes of slide until 5/5 when a rise of  $3^{\circ}$ – $4^{\circ}$  killed line XIIa, XIIb being ended at the same time. The accompanying graphs show that endomixis occurred on the aver-



age once in every twenty-one generations in lines XIIa and b, while in the parent line XII, it occurred once in twenty-six generations, a decrease of five generations in favor of the former lines.

*Experiment 13* (figs. XIII and XIIIa)

In this experiment line XIII was branched off from line IV on 5/19 and carried through seventy-nine (eighty) generations<sup>13, 14</sup> with daily slide changes until 6/11 without the occurrence of endomixis. On 5/25 line XIIIa was branched off and carried, with daily slide changes, until its death on 5/28, endomixis ensuing on 5/26 four generations after the transfer to the higher temperature occurred.

<sup>13</sup> On 6/7 when the slide was changed only three cells were present and as it was impossible to tell which of these belonged to the first and which to the second generation of daughter cells, the total count is uncertain to the extent of one generation.

<sup>14</sup> Reckoning from the last endomixis in line IV.

*Experiment 14 (fig. XIV)*

On 5/25 line XIV was branched off from line IV and carried with daily slide changes through thirty-two generations until 6/5. Endomixis occurred on 5/26 and 6/3 with an intervening period of twenty-one generations as compared with one of seventy-six in the parent line.

*Experiment 15 (fig. XV)*

On 6/6 line XV was branched off from line XIII and carried through thirty-six generations with daily slide changes until its death on 6/18. Endomixis occurred on 6/7 and 15 with an interval of twenty-six generations, as compared with a period of seventy-nine (eighty) generations<sup>13, 14</sup> without endomixis in the parent line.

*Experiment 16 (fig. XVI)*

In this experiment line XVI was branched off from line IIa on 5/29 and carried through sixty-one generations with daily slide changes until its death on 6/18. Endomixes occurred on 5/30, 6/8 and 6/17 with intervening periods of thirty-three and twenty-five generations respectively, while in the parent line, IIa, seventy-eight generations intervened between two successive endomixes.

*Experiment 17 (fig. XVII)*

Line XVII was started from line VI on 5/29 and carried through three generations only with daily slide changes until its death on 6/1. Endomixis ensued on 5/30 with no generations before its occurrence, while in the parent line there were thirty-seven generations before its next occurrence.

Conversely I have tried the effect of lowering the temperature from room temperature (approximately 20°), and from 30° to 14°. In none of the few experiments of this character which I have performed have I succeeded in inducing endomixis. I have not however carried the experimental lines through a sufficient number of generations to ascertain whether or not lowering the temperature would influence the frequency of its occurrence.

I have also performed a number of experiments with various chemicals (ammonia, alcohol, urea; strychnine and nicotine), but in no case have I succeeded in definitely inducing endomixis or increasing its frequency. In most of these experiments, however, the race has not lived long enough to enable me to make any final statements regarding the influence of such experiments on endomixis.

The discussion of these results may best be postponed until further evidence has been presented bearing on the second question above stated, namely: Is endomixis an evidence of depression or a means of rejuvenation of a depressed race? These two hypotheses are not necessarily alternate to each other. Endomixis may be both an expression of, and a means of relief from a depressed condition. The hypothesis of Hertwig, Popoff and others, according to which nuclear reorganization in *Paramecium* is a parthenogenetic process is based on the generally accepted interpretation of conjugation, with its accompanying nuclear reorganization, as a rejuvenating process. Jennings ('13) however, and more recently Mast ('16) have shown that in *Paramecium* and *Didinium* this interpretation is very doubtful, while Woodruff's well known culture of *Paramecium aurelia* has shown that a race of Protozoa may live indefinitely without this process. Woodruff and Erdmann ('14, '16) however maintain that the longevity of their race is due to the constantly recurring nuclear reorganization or endomixis, and suggest that rejuvenation occurs in conjugation also by virtue of the similar process taking place at that time.

The evidence for this interpretation is the occurrence of low points in the division rate coincident with the reorganization process, following which the division is restored to its normal rate. This evidence however is proof merely of a certain correspondence between depression periods and endomixis, and is no more indication of a rejuvenating than of a depressing effect of the latter.

In my own experiments described and plotted above, I have found that accompanying endomixis there is almost invariably a temporary slowing down in the division rate. An exception is however seen in line III, 6/13-6/15 (fig. III), where the process is associated with a rising rather than a falling division rate. This is also seen in the exception noted by Woodruff and Erdmann to which I have already referred.<sup>15</sup>

I have further found several instances of temporary reduction in the division rate unaccompanied by endomixis. Cases of

<sup>15</sup> See page 35.

this are seen in lines Ia, 6/1 and 6/6 (fig. Ia), and III, 5/28-6/1 (fig. III).

While endomixis is usually followed by restoration of the division rate, this is not invariably the case, line IIa, 6/15-6/17 (fig. IIa). Exceptions to this rule are especially noticeable in those lines which were carried in stale culture media—II, 3/5-3/7, 5/13 (fig. II), VIIb, 2/19, 2/20, 3/24-3/25 (fig. VIIb), etc.

In order to test the hypothesis that endomixis is a sign of depression I have performed a series of experiments, using the death rate as a criterion of depression.

When transferred from a culture in which they are abundant to a new hay infusion, I have in several instances been unable to obtain good cultures of *Paramecium* and it has only been after one or more trials that I have succeeded in obtaining healthy cultures. I have further found that in isolating single specimens on cover glasses in a few drops of hay infusion a large per cent of such individuals die. The *Paramecia* are visibly sensitive to such changes in their environment. When a specimen is placed in a few drops of a new culture fluid, the old culture can usually be recognized in the new by a difference in color. When the animal in swimming about in the old fluid comes in contact with the new it reacts vigorously just as it does when brought in contact with a salt solution or other harmful substance, and if it crosses the boundary into the new medium it swims violently about showing in the plainest manner its sensitiveness to the change. The changes moreover to which the animals respond so vigorously are apparently slight, the culture media usually consisting of the same substances (meadow hay and tap water) so that the differences between them consist in age, with consequent chemical change, and in the substances given up to the old medium by its inhabitants themselves.

An exact physico-chemical and biological analysis of a *Paramecium* culture would be, to say the least, a very serious problem; and no one has yet, so far as I know, undertaken it, although Peters ('04, '07 a and '07 b), Woodruff ('11 and '12) and Fine ('12) have made a beginning in this very interesting field.

In the present study I have made a sufficient comparison of the osmotic pressures of several cultures used in isolation experiments to show that the differences are probably too slight to explain the high death rate which is found in many cases. In order to ascertain whether there is any close numerical correspondence between the percentage of loss in such isolation experiments and the percentage of multinucleate specimens in the culture at the time of isolation seven experiments were performed. In each of these the *Paramaecia* were isolated with a capillary pipette, and placed on cover glasses on slides in a small slide stack, which was kept in a moist chamber. The exact amount of isolation fluid was not measured. It was, however, in every case many thousand times the volume of one *Paramaecium* and sufficient to maintain many individuals. The kind of culture fluid used varied in the different experiments. In experiment 18 an old hay infusion in which *Paramaecia* had been living for some time was employed. In experiment 19 an infusion of grass and in 20 and 21 a fresh infusion of hay was used. In experiment 23 and part of 22 a sterilized 0.025 per cent solution of Liebig's extract of beef in distilled water was used and in 24 and part of 22 the parent culture (hay infusion). The number of *Paramaecia* which died within about forty-eight hours was noted, which number gave the percentage death rate. A number of individuals chosen at random from the culture to be tested were stained and mounted, from which the percentage of multinucleate specimens was determined. The number so chosen varied in different experiments but was sufficiently large in each to give a fair estimate of the latter figure. Further details of these experiments may be omitted, the results being summarized in the following table.

In only two (18 and 19) of these seven experiments is there any correlation between the death rate and the percentage of multinuclearity at the time of experiment and this is probably accidental. The objection may be brought against these experiments that different cultures were employed in different cases and therefore comparisons are misleading. What I believe I have shown, however, is that there is no relation between death

EXPERIMENT	PER CENT DEATH RATE	PER CENT MULTINUCLEATE SPECIMENS
18	50.0	46.5
19	48.0	42.6
20	98.0	14.6 <sup>16</sup>
21	60.0	16.3 <sup>16</sup>
22	45.5 <sup>17</sup>	57.1 <sup>19</sup>
	16.7 <sup>18</sup>	
23	80.0	20.0 <sup>20</sup>
24	00.0	58.1

rate and percentage of multinuclearity regardless of the kind of culture used in the isolation experiments. This objection moreover will not hold in regard to comparisons between those experiments in which the same culture media were employed (compare experiment 20 with 21 and 22 with 23 and 24).

These experiments further demonstrate the interesting fact that there is no correspondence between death rate and multinuclearity even when the specimens are isolated in the parent culture (compare experiments 22 and 24).

In most cases of the death of *Paramaecium* in isolation cultures in which I have examined the nuclear condition of the dead animals I have found it mononucleate, further proving the absence of a positive correlation between multinuclearity and death.

What function then may be ascribed to endomixis? It is evidently a necessary process in the life of the race, occurring at more or less regular intervals. The fact that in the experiments above described the frequency of multinuclearity was markedly increased by impurity in the culture medium and by increase of temperature; conditions which, on the one hand subjected the animals to the influence of metabolic end products,

<sup>16</sup> These percentage counts were made from the same culture—in experiment 20, five days before and in experiment 21 two days after the isolations.

<sup>17</sup> In parent culture.

<sup>18</sup> In beef extract.

<sup>19</sup> Percentage count made from slides prepared one and two days preceding the isolations.

<sup>20</sup> Percentage count made from slides prepared one and two days after the isolations.

and on the other increased the rate of their own metabolism; supports the view of Woodruff and Erdmann as to its rejuvenating function, and suggests that the macronucleus may serve in part as a reservoir of waste products which are distributed through the cells in endomixis and can then be excreted through the contractile vacuoles. The action of distilled water is not clear. It may possibly be a starvation effect, as the number of bacteria are materially reduced in these cultures. It may on the other hand be a toxic effect due directly to the action of the distilled water. The latter hypothesis is supported by the result in experiment 10, where the animals were not killed so long as there was a trace of the old culture on the slide, but died almost immediately after washing in, followed by transfer to pure distilled water.

This interpretation is supported by the fact that *Paramecium* will not withstand direct transfer to distilled water, but may be adapted to it to a certain extent by increasing the dilution of the culture gradually, and by the fact that in one instance (Xb-4/11) the race died out while numerous food vacuoles were present. It also agrees with the work of previous investigators.

That a temporary reduction of growth should be associated with endomixis is only to be expected in view of the great changes taking place at this time within the cell. *Paramecium* can however undergo brief depression periods without the intervention of endomixis, as proven by the graphs of Woodruff and Erdmann and by my own results in this investigation; while endomixis does not necessarily rejuvenate as shown by my results in experiments 2, 7 and 8. These facts show that other factors than endomixis are involved in determining the division rate of *Paramecium*. In the case of some of the experiments, notably 7 and 8, where occasionally several days ensued without any division, it is possible, though not probable that endomixis occurred more frequently than I have recorded it. Even so, however, my conclusions would be emphasized rather than negated thereby. Whether we may consider endomixis as a reproductive, as well as a rejuvenating process, or consider it the equivalent of parthenogenesis in the Metozoa,

is a question upon which we have as yet no certain evidence. Until the question of the function of conjugation in Infusoria is cleared up, that regarding the relation of endomixis to parthenogenesis may perhaps better be held in abeyance.

#### CONCLUSIONS

1. Endomixis in *Paramecium* can be experimentally induced, especially by the use of metabolic end products and by increasing the rate of metabolism of the animals themselves.

2. It is not a lethal process, but may be associated with a temporary depression.

3. It is more or less cyclical in character and probably has a rejuvenating function.

4. It is not however a necessary accompaniment of temporary depressions and may not be followed by rejuvenation. Other factors are concerned in determining the division rate.

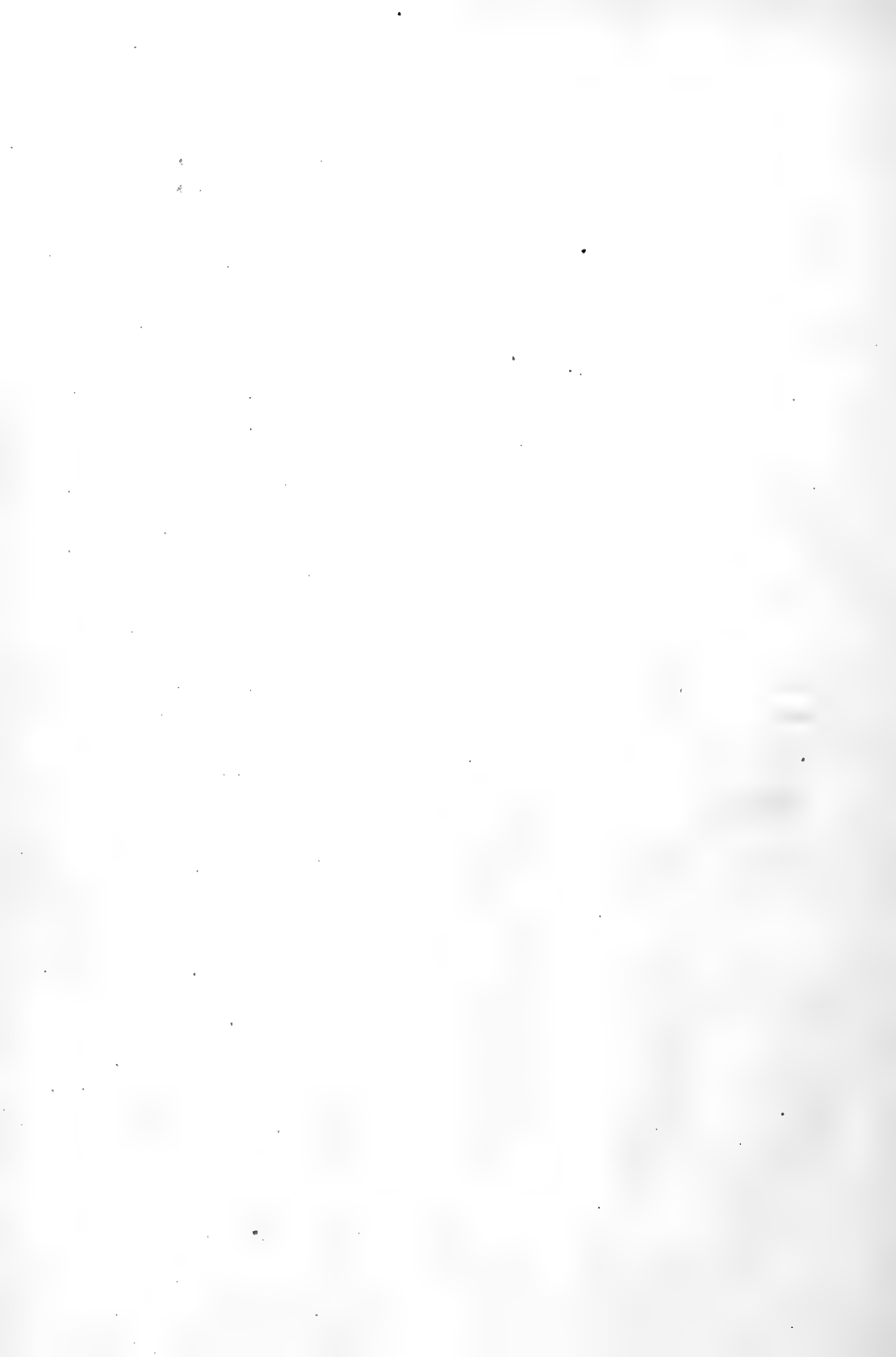
5. Its relation to parthenogenesis is not clear.



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<sup>21</sup> Not seen by me.



# METABOLIC GRADIENTS IN AMOEBA AND THEIR RELATION TO THE MECHANISM OF AMOEBOID MOVEMENT

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FOURTEEN FIGURES

## I. INTRODUCTORY REMARKS

The existence of a differential axial susceptibility to certain substances has now been demonstrated for a number of adult and embryonic organisms, including algae, representatives of most of the groups of lower invertebrates, and embryos of echinoderms, annelids, and vertebrates.<sup>1</sup> This work has shown that the anterior end, head, or apical region of these organisms is most susceptible to lethal concentrations of the substances in question and that this susceptibility decreases along the principal axis of symmetry. In some cases it has been possible to demonstrate differential susceptibility along other axes also, as the ventro-dorsal axis, (dorso-ventral in vertebrates) and the medio-lateral axis (Child, '15, Chap. III).<sup>2</sup> These gradients are the cause of the formation of anterior or apical ends, and of axes of symmetry and not the result of them. This phase of the matter cannot be discussed here but is treated to some extent in Child ('15).

<sup>1</sup> A general discussion of metabolic gradients will be found in Child ('15, Chap. III), with references to papers published up to that time; since then, the following papers on gradients have appeared: Hyman ('16 a), Child ('16 a, '16 b, '16 d, '17 a, '17 b).

<sup>2</sup> Methylene blue has proved to be a particularly favorable reagent for the demonstration of these minor axes. Thus in oligochaetes, killed in methylene blue, it can plainly be seen that the disintegration proceeds faster along the ventral side than along the dorsal side, and further that disintegration first occurs in the ganglia of the ventral cord, indicating that the gradient has its chief seat in the nervous system.

These gradients are demonstrable by several direct and indirect methods. The chief direct method consists in the exposure of the organism to solutions of certain substances of sufficient concentration to kill it within a few hours; the differences in time of death of the various levels of the body are then observed. A number of reagents of widely different chemical composition have been used for this purpose—potassium cyanide, several substances belonging to the class of narcotics, acids, alkalies, some salts, and one or two vital dyes—and all of them yield essentially the same death gradients. When colored substances are employed a beautiful color gradient may often be demonstrated in the organism before its death, the most susceptible regions becoming more deeply colored. Thus the mixture of dimethyl-*p*-phenylene-diamine and  $\alpha$ -naphthol yield within the animal a blue precipitate of indophenol, the amount of precipitate showing a definite and well-marked gradation along the antero-posterior axis. Gradients in organisms can also be demonstrated by means of a sensitive galvanometer, although the method has never been employed for this purpose intentionally. Mathews ('03) records that the head of coelenterate polyps is electro-negative<sup>3</sup> to the stem, and anterior levels of the stem negative to posterior levels; and Child ('14) later showed that in these forms anterior levels are always more susceptible to the reagents mentioned than posterior levels. Morgan and Dimon ('04) repeated Mathews' experiment, using the earthworm, and found that the anterior and posterior ends of this animal are electro-negative to the middle, and I demonstrated that in annelids in general the anterior and posterior ends are more susceptible to cyanide than the middle regions (Hyman, '16 a). In this connection it is worthy of remark that most Protozoa when subjected to an electric current orient themselves so that their anterior ends are directed toward the cathode and swim to the

<sup>3</sup> Electro-negative with regard to the external current; in reality anterior levels are electro-positive to posterior ones. It is very unfortunate that it has become customary to refer to the bioelectric currents according to the direction in which the current passes through the galvanometer and not in which it passes through the tissue.

cathode. Since the anterior ends of Protozoa are more susceptible to cyanide than other levels of the body, it is practically certain that a difference of potential exists in them like that discovered by Mathews for coelenterates, and that therefore their anterior ends are electro-positive (internally) to the rest of the body. This would account very simply for their behavior in the electric current. The earthworm may be said to be especially devised by nature to furnish a crucial test for this explanation. It has recently been found that when the earthworm is subjected to the current, it bends itself into a U-shape and travels toward the cathode with anterior and posterior ends directed to the cathode and middle region directed to the anode. Clearly a fundamental relationship exists between electrical currents in living matter and susceptibility; and it is reasonable to suppose that both are referable to the same cause.<sup>4</sup>

The axial gradients may also be demonstrated indirectly by the use of proper non-lethal concentrations of the same substances as are employed in the direct method. Differential inhibitions and accelerations of growth, development, regeneration, etc., along the principal axes are thus produced. Particularly illuminating are the experimental modifications of development, resulting in a great variety of predictable teratological embryos and larvae (Child, '16 c and much unpublished work).

Regarding the explanation or interpretation of the susceptibility gradients, a few words must suffice here, as this matter has been discussed at length in many of Child's papers. The

<sup>4</sup> The existence of these permanent differences of potential along the axes of organisms (they have long been known in plants, also) can scarcely be explained on the basis of the depolarization theory of Lillie ('14, '15, '16) and others. According to this theory, stimulation causes a local change in the electrical condition of the surface (a depolarization of the surface); the stimulated area thus becomes electrically different from neighboring unstimulated regions, and this difference of potential is the cause of the origin of an electric current, flowing (externally) from the unstimulated to the stimulated region. According to this idea, all bioelectric currents originate in this way. I am unable to see how this theory could be applied to permanent differences of potential such as I am referring to; they almost certainly are of metabolic origin, and are the incidental result of the metabolic gradient. Their similarity to other bioelectric currents would indicate that all such currents originate in metabolic (i.e., chemical) differences and are merely incidental to the presence of such differences.

susceptibility gradient is a gradient in metabolic rate, that is, the regions most susceptible to poisons are those where the metabolic reactions occur with the highest speed. The fact that a great variety of substances will give a death gradient shows clearly that their action cannot be specific but that all affect the same general fundamental process in the protoplasm. And this process is that complex we designate as metabolism since all factors which are known to modify the metabolic rate also modify the susceptibility to these substances and in the same direction. This relation between susceptibility and metabolism is further confirmed by direct determinations of carbon dioxide output in a Tashiro biometer or by an indicator (as the phenolsulphonephthalein colorimetric method of Haas, '16); those parts of an organism most susceptible to cyanide and other substances give off the most carbon-dioxide, i.e., they are carrying on metabolic processes at a higher level. Similarly the precipitation of indophenol is a direct measure of oxygen consumption, and is greatest in those regions which are most susceptible. Some of the susceptibility reagents may affect metabolism directly—potassium cyanide almost certainly does so (cf. Hyman, '16 b)<sup>5</sup>—while others may act indirectly. The existence of axial electric currents in organisms confirms our general conclusion since bioelectric currents are invariably associated with increased functional activity, as nerve conduction, muscle contraction, gland secretion, etc. Furthermore the region of increased activity is always electro-negative (externally) to non-active regions. If, then, the anterior or apical end of organisms is more active metabolically than posterior levels, it should be electro-negative to them, as is exactly the case.

<sup>5</sup> A consideration of the structure of potassium cyanide suggests why it has such a powerful effect on protoplasm. Potassium cyanide is not really a cyanide, but an isocyanide, that is, its structural formula is  $K - N \equiv C$ . It therefore contains bivalent carbon, one of the most reactive atoms known to chemistry, since it has two unoccupied valences which unite with the greatest avidity with many atoms and radicals, or in lieu of those with other molecules of cyanide. Bivalent carbon unites with oxygen with extreme readiness, and potassium cyanide is in fact one of the most powerful reducing agents known; hence, its effectiveness in depressing and inhibiting oxidations in protoplasm and elsewhere.

For these reasons, the organic gradients are generally designated as metabolic gradients, although in most cases what we really determine experimentally is the relative susceptibility of various parts of the organism to substances which are toxic or which are used in toxic concentrations.

As the previous work had been done on axiate organisms it seemed to Professor Child and myself that it would be interesting to try susceptibility experiments on an organism such as amoeba which has no permanent axis. I have used two strains of large amoebae, one grown on hay culture, the other on wheat.<sup>6</sup> Both of these strains are of the granular type and both seem to correspond to the description of *Amoeba proteus* although they are distinctly different in appearance and behavior. I shall have occasion to refer to these differences later. Most of the susceptibility experiments were performed on the hay variety.

## II. SUSCEPTIBILITY EXPERIMENTS

Potassium cyanide was the reagent employed. It was, of course, essential that the pseudopodia should remain extended during the action of the cyanide if any differential susceptibility was to be detected. This at first seemed unattainable as the

<sup>6</sup> Hay culture: prepared according to the method of Parker ('15). After the culture has stood for one week, a small amount of stale white bread is added, and the culture is inoculated with the amoebae. The culture is successful only when a brown scum appears in it. Wheat culture: boil some wheat for five minutes in a small quantity of water, and put the boiled wheat into jars of water in the proportions of not more than one gram of wheat (dry weight) to a liter of water. Inoculate with unicellular green algae and with amoeba. The amoebae may appear abundantly in a week or two after starting the culture but soon disappear and a permanent and rich culture of amoebae is obtained only after the green algae have established a luxuriant growth, about one month from the start of the culture. The amoebae are always on the bottom of such a culture, and continue in abundance for a long time. The life of the culture may be almost indefinitely prolonged by removing some of the water and adding fresh water and a little fresh boiled wheat from time to time. Both the varieties of amoeba were obtained from the Des Plaines river, an old and muddy stream, which receives considerable sewage, near Lyons, Ill. I have now abandoned the hay method of culture, and raise both kinds on wheat. This shows that the differences between them are not due to differences in the culture media, since both occur simultaneously in the same jar, although the optimum conditions are not quite the same for the two.

amoebae withdrew their pseudopodia when placed in cyanide solutions of the concentrations which we had been accustomed to employ. This difficulty was surmounted by resorting to very strong solutions of cyanide and the following procedure was adopted. The amoebae were mounted on a slide in a drop of water, a single, favorable specimen selected, its form, position of pseudopodia, direction of movement, etc., carefully noted, and then, while it was being observed under the low power, a drop of strong cyanide solution was allowed to fall upon it from a pipette. Movement ceases instantaneously with the pseudopodia fully extended, and in a few seconds death and disintegration begin.

The potassium cyanide was weighed out fresh for each experiment with some care but not with quantitative precautions so that the concentrations as stated are only approximate. Furthermore, in stating the concentration employed, I have taken no account of the dilution of the solution due to the water in which the amoeba is mounted. The amoeba is therefore really exposed to a concentration somewhat less than that which is stated. In point of fact, I have made definite mention of concentration only in order to give some idea of about the strength required since the effect of a given solution varies greatly with the temperature and with the amoebae themselves. The experiments described below were always performed at room temperature (about 21 or 22°C.), as I found it impractical to work on cold days or after a cold night, but even so, the variations in the organisms are so considerable that one must in practice make up a strong solution, say molecular, and then dilute it until the desired effect is obtained.

Observations were made with a Leitz objective no. 4 and ocular no. 4. Drawings are necessarily free-hand, and hurriedly done as the disintegration occurs with great rapidity.

1. *Experiments with molecular or  $\frac{1}{2}$  molecular KNC.*<sup>7</sup> When a drop of cyanide of this strength falls upon the moving amoeba,

<sup>7</sup> Solutions of cyanide of this strength are of course strongly alkaline owing to the slight degree of ionization of hydrocyanic acid. This condition however is not objectionable because alkali alone gives a death gradient identical with that in cyanide. Of course, death occurs more quickly owing to the fact that



it ceases to move and remains motionless, neither extending nor withdrawing its pseudopodia. The first change that occurs is one that was totally unexpected and for a time seemed inexplicable. A few seconds after the cyanide has been applied, the ectoplasm at some spot on the surface dissolves away, and the fluid endoplasm instantly bursts out and mingles indistinguishably with the surrounding water. The point in the ectoplasm where this occurs bears no relation to any of the pseudopodia but it is almost invariably at the 'posterior' end of the amoeba, i.e., the end opposite the direction of locomotion; it does, however, occasionally take place on the side of one of the larger pseudopodia. As a result of the loss of some of the endoplasm, the amoeba shrinks considerably, and changes its form to some extent but not sufficiently to interfere with the identification of the pseudopodia.

This entire initial behavior of the amoeba to strong cyanide unavoidably suggests that the ectoplasm is a firm elastic layer inclosing a fluid, the endoplasm. Since the ectoplasm is in a state of tension, the endoplasm bursts forth immediately when the ectoplasm is locally ruptured. The explanation of the local rupture of the ectoplasm is deferred until the next section where the action of slightly weaker solutions of cyanide is described.

Shortly after the ectoplasm breaks disintegration sets in. Disintegration invariably begins at the tips of the pseudopodia, and proceeds rapidly down each of them to the posterior end of the amoeba in the case of elongated forms, or to the central part in the case of radiate forms. In general, where several pseudopodia are present, the most recent or most recent vigorous pseudopodium disintegrates first and the others follow in the reverse order of their formation. In the process of disintegration the ectoplasm appears to dissolve away so that there is no longer any boundary between the endoplasm and the water, and the endoplasm then bursts forth, and spreads into the water. All that remains visible of the amoeba is a scattered collection of the

alkali increases permeability. Thus Child has found that addition of alkali to the cyanide (in dilutions of cyanide too weak to be in themselves alkaline) greatly increases the susceptibility of *Planaria* while addition of acid decreases it.

endoplasmic granules; but upon moving a needle point about among these granules one finds that they are contained in a viscous substance. I have not been able to observe the fate of the nucleus but as it is never to be found among the extruded granules, I conclude that it disintegrates with the protoplasm.

Figure 1 represents four stages in the disintegration of an amoeba in molecular KCN. In *a* the amoeba is flowing in the direction indicated by the external arrow; the posterior end presents the corrugated appearance which is an invariable characteristic of the amoebae with which I have worked and which I shall refer to later. After the application of the cyanide, the ectoplasm bursts at the point *x* in *b*, some of the endoplasm



Fig. 1 Disintegration of amoeba in molecular KCN. *a*, the amoeba is flowing in the direction of the external arrow; *b*, after application of the cyanide, the ectoplasm bursts at *x*; *c*, the endoplasm escapes from the rupture; *d*, disintegration has begun at the distal end of the pseudopodium and is proceeding as indicated by the internal arrow.

flows out, and the amoeba contracts slightly as a consequence. The endoplasm continues to stream forth for a short time as shown in *c*. Next the tip of the pseudopodium begins to disintegrate and the disintegration proceeds down the axis of the pseudopodium until it reaches the previously extruded mass of granules at the posterior end. This stage is shown in *d* where the internal arrow represents the direction of disintegration.

Figures 2 and 3 illustrate the same stages in the disintegration of two other individuals. Figure 4*a* is an amoeba with several slender pseudopodia, which are numbered in the order of their formation, 5 being the most recently formed, and 1 the oldest. After exposure to molecular cyanide, the ectoplasm bursts in the usual way at the non-active end of the animal (4*b*); then disintegration begins at the tips of the pseudopodia, first in pseudopo-

dium 5, then 4 and 3 simultaneously, then in 2, and finally in 1 (fig. 4c). The internal arrows indicate the further direction of the disintegration. The disintegration gradients appear very clearly in such individuals with long slender pseudopodia; each pseudopodium gradually melts away from its distal to its proximal end. Such individuals are also favorable for the observation of the relation between recency of formation and time of disintegration of a given pseudopodium, the youngest pseudopodia dying first.



Fig. 2 Disintegration of amoeba in molecular KNC. *a*, amoeba advancing with two pseudopodia; *b*, after exposure to cyanide, the ectoplasm bursts at the posterior end; *c*, disintegration from distal ends of the two pseudopodia inwards.



Fig. 3 Similar to figures 1 and 2. *a*, normal animal with two advancing pseudopodia; *b*, rupture at posterior end on exposure to cyanide; *c*, disintegration of the pseudopodia.



Fig. 4 Disintegration of an amoeba with five slender pseudopodia, 1 the oldest, 5 the youngest; the animal is advancing along pseudopodium 5 in *a*. *b*, after exposure to molecular KNC, the ectoplasm ruptures at the posterior end; *c*, disintegration in the pseudopodia, first in 5, then in 4 and 3; 2 followed shortly after, but 1 disintegrated more slowly.

2. *Experiments with  $\frac{1}{3}$  or  $\frac{1}{4}$  molecular KNC.* By the use of these concentrations I have been able to discover the cause of the rupture of the ectoplasm which has been described in the preceding section as the initial effect of the cyanide. Upon the application of  $\frac{1}{3}$  or  $\frac{1}{4}$  molecular cyanide, the animal becomes motionless as before, but very soon it can be seen that a pseudopodium is beginning to form. This pseudopodium nearly always starts from the posterior end of the amoeba, although sometimes it occurs on the side of a large pseudopodium, that is, in exactly the same places where the ectoplasm first breaks when the stronger concentrations of cyanide are employed. So soon as this pseudopodium appears, it ruptures, and the endoplasm flows out in the same manner as already described; and following these the previously existing pseudopodia disintegrated from their tips inward exactly as when molecular KNC is used.

As a result of this experiment, which has been repeated a great many times, I have come to the following conclusion. The response of the amoeba to the strong stimulation of the cyanide is pseudopod formation, and pseudopod formation at the least active part of its body, the part which would be least affected by the reagent. In cyanide of molecular strength, the pseudopodium does not and can not form but the ectoplasm ruptures at the place where the pseudopodium would appear. This is demonstrated by the fact that in slightly less concentrated solutions a pseudopodium actually does appear in exactly the same places where the ectoplasm breaks in molecular KNC. Since, however, no pseudopodium appears in the latter case one is forced to conclude that some internal change precedes pseudopod formation, and that this internal change is the same as that which makes protoplasm in general more susceptible to cyanide than it previously was, that is, a metabolic change. This internal change occurs in both concentrations of cyanide, the susceptibility is locally increased, the ectoplasm dissolves away, but in the one case this occurs before a pseudopodium forms while in the other the pseudopodium appears. I believe the observations permit no other interpretation; and the conclusion is further confirmed by the experiments on the 'shock' reaction of amoeba to be described later.

Figures 5, 6, and 7 are drawings of the disintegration of amoeba in  $\frac{1}{4}$  molecular KNC. In 5*a* the amoeba is flowing in the direction indicated by the external arrow. After exposure to the cyanide, the animal contracts slightly and sends out a pseudopodium from the posterior end at *x* (fig. 5*b*). In 5*c* the newly formed pseudopodium has ruptured, and in 5*d* disintegration has begun at the tip of the old pseudopodium and is proceeding



Fig. 5 Disintegration of amoeba in  $\frac{1}{4}$  molecular KNC, *a*, amoeba flowing in direction of external arrow; *b*, after application of the cyanide, a pseudopodium flows out at the posterior end at *x*; *c*, this new pseudopodium ruptures; *d*, disintegration from the distal end of the pseudopodium present in *a* inwards.



Fig. 6 Same as figure 5. *a*, amoeba flowing in direction of arrow with pseudopodium 1; the amoeba has abandoned pseudopodium 1, and is advancing with pseudopodium 2; on exposure to  $\frac{1}{4}$  molecular KNC, a pseudopodium forms at the posterior end at *x*; *c*, pseudopodium at *x* has ruptured; *d*, disintegration from tips of pseudopodia present in *a*, pseudopodium, 2, the most recently active one, preceding.

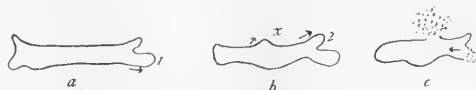


Fig. 7 Disintegration in  $\frac{1}{4}$  molecular KNC. *a*, amoeba flowing with pseudopodium 1; *b*, exposure to cyanide; amoeba now advancing with pseudopodium 2, sends out a pseudopodium at *x*, from the side of the body; *c*, pseudopodium at *x* ruptures, disintegration beginning from tip of pseudopodium 1. Although pseudopodium 2 was more recent, pseudopodium 1 was much more vigorous.

towards the posterior end. Figure 6 is similar to figure 5. Figure 7 is one of those infrequent cases where the pseudopodium formed after the application of the cyanide arises at the side of the body and not at the posterior end.

3. *Experiments with  $\frac{1}{6}$  to  $\frac{1}{10}$  molecular KNC.* When exposed to these concentrations, the amoeba begins to contract slowly. In this contraction the ectoplasm becomes greatly wrinkled and corrugated. There is no attempt at pseudopod formation. The old pseudopodia are recognizable for some time as blunt projections. If disintegration occurs while the pseudopodia are still identifiable, it involves the pseudopodia first as usual. Generally, however, disintegration takes place only after the

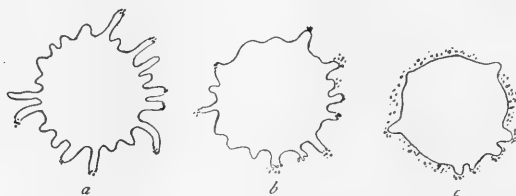


Fig. 8 Disintegration of amoeba in  $\frac{1}{10}$  molecular KNC. *a* shows corrugated form assumed by the amoeba in this dilution; *b* and *c*, disintegration of the short pseudopodia.

amoeba has contracted to a spherical mass from the surface of which there project numerous short pseudopodia which are really the corrugations produced by the contractions (fig. 8*a*). In this condition all trace of the previously existing pseudopodia is lost. In the disintegration of these spherical masses, the surface corrugations gradually melt away, the longer and more slender ones first, the disintegrated protoplasm remaining as little droplets (fig. 8). The entire surface of the sphere then dissolves away simultaneously, and the protoplasm by a sudden expansion which is almost an explosion spreads out into the surrounding water. The entire process requires some twenty to forty minutes.

4. *Experiments with more dilute solutions.* In dilutions somewhat greater than  $\frac{1}{10}$  molecular, the amoeba gradually assumes the spherical form studied with short pseudopodia as in the pre-

ceding case. Here, however, the pseudopodia are not fixed, but continue to change slowly, new ones being formed, and old ones retracted. The animal thus slowly alters its shape but does not change its position. After this condition has persisted for several hours, the pseudopodia are gradually completely withdrawn, leaving the surface smooth and free from corrugations. In this condition death occurs, generally by simultaneous rupture of the entire surface. Sometimes a local rupture precedes the general disintegration but in such cases it is impossible to relate this occurrence to any preceding condition of the pseudopodia.

In very dilute solutions,  $\frac{1}{50}$  molecular or above, the amoeba moves about in the normal manner for many hours although more slowly than usual. Eventually the movement ceases; the animal contracts into a smooth sphere, entirely free from surface irregularities. The amoeba remains in this state for hours, bearing considerable resemblance to an encysted individual except that a protecting membrane is lacking. I have not observed the death of the animals in these dilutions but have found the disintegrated remains.

### III. INTERPRETATION OF RESULTS; THE SHOCK AND AVOIDING REACTIONS

These experiments demonstrate that a local region of increased susceptibility exists before pseudopod formation; that a differential susceptibility exists along the axis of each pseudopodium from its distal to its proximal end, the distal end being most susceptible; that the susceptibility of a pseudopodium depends upon its age and vigor, the youngest and most vigorous being most susceptible, while old non-functioning pseudopodia are much less susceptible, although still exhibiting differential susceptibility. These are the results as they stand without interpretation. The interpretation here of course must be the same as that made for susceptibility to toxic agents in general. No one will assume that the amoeba is different from other organisms in this respect. Now as I have already pointed out in my introductory remarks there is a clear relation between susceptibility and metabolic rate, such that regions of high metabolic rate

are more susceptible than those of lower rate. It is futile and unnecessary to inquire whether this relation is direct or indirect, owing to our ignorance as to the way in which toxic agents act on protoplasm. The evidence is in my opinion quite sufficient to warrant the conclusion that a differential susceptibility indicates a differential metabolic fate. The results may then be translated into metabolic terms as follows: a local increase in metabolic rate occurs, resulting in the beginning of a pseudopodium, and the protoplasm continues to flow out as long as the metabolic increase persists with its original intensity at the advancing tip. A metabolic gradient thus results along the axis of each pseudopodium. As soon as the metabolic rate at the distal end of the pseudopodium falls below a certain value, movement ceases, as shown by the fact that non-growing pseudopodia are less susceptible to cyanide than moving ones. In contemplating these phenomena one is irresistably reminded of the metabolic 'blaze' which accompanies (precedes?) muscle contraction, nerve conduction, gland secretion and protoplasmic activities in general.

One may say then that metabolic gradients exist in the amoeba as in other organisms but that these gradients are temporary and readily appear and disappear. The advancing end or ends of the amoeba have the highest metabolic rate of any part of the organism and hence correspond to the anterior ends of other organisms. Clearly in the amoeba an anterior end is differentiated physiologically but not structurally; and this physiological differentiation if it should become permanently established would obviously be a basis for a morphologically differentiated head. Thus the metabolic gradient is the cause of the antero-posterior axis, and even in the amoeba its presence at once brings about a certain structural gradation in the physical condition of the protoplasm, as will appear later in this paper.

I am aware that various criticisms can be made against this interpretation of the experimental results. I am unable, however, to conceive of any explanation of the observed facts which must not postulate an internal difference of some sort along the axis of the pseudopodium. Be this postulated difference one of



permeability, or of colloidal state, or what-not, it must be accounted for, and accounted for on the basis of some internal change in the amoeba. It might be said that the tips of the pseudopodia are more freely exposed to the cyanide than other parts, and that therefore the cyanide penetrates them more rapidly. This objection is invalid, not only because experience indicates that the cyanides penetrate readily into protoplasm but for the reasons that susceptibility differences exist between pseudopodia which are equally exposed to the solution and that the most susceptible places are not the pseudopodia but those places in the central mass where the pseudopodia are about to appear. It might further be said that differences in permeability or in physical state along the axis of the pseudopodium would account for the differential disintegration. I believe such differences do and must exist; in fact I think that amoeboid movement is directly due to alterations of the colloidal state but I am unable to account for such alterations except as the result of internal metabolic changes.

Criticism may be made of my explanation that the initial rupture of the ectoplasm in normal cyanide is due to an attempt at pseudopod formation, because if the cyanide is diluted slightly, an actual outflow of protoplasm precedes the rupture. It might be said that this outflow is not a pseudopodium but merely a stage in the bursting of the ectoplasm. In support of this contention one might maintain that the amoeba does not ordinarily put out pseudopodia at its posterior end, where this rupture of the ectoplasm generally takes place. It is well known to all observers of amoeba that the animal tends to continue in the same direction for long periods of time, pursuing a somewhat zigzag course, the pseudopodia advancing first on one side and then on the other side of the anterior end. It retains the same anterior end as long as undisturbed and is not ordinarily observed to stop suddenly in its course and put forth a pseudopodium at its posterior end. These considerations have impelled me to perform some experiments on the behavior of amoeba.

Jennings ('04, '06) has described the reactions of amoeba to various stimuli. His general conception of the avoiding reaction

is that it is only partially determined by the localization of the stimulus and the part of the body on which the stimulus acts and that internal differentiation plays an important rôle in determining the direction of the subsequent movement. To quote directly:

But the localization of the external stimulus is not the only factor in determining the direction of locomotion. . . . After stimulation at one side or end, the new pseudopodium is as a rule not sent out in a direction exactly opposite that from which the stimulation comes. It usually appears, as we have seen, on some part of the original anterior end of the body, and at first alters the course only slightly. . . . If the new direction still leaves the anterior part of the body exposed to the action of the stimulus, then a new pseudopodium is sent out in the same way, still further altering the course. . . . From these facts it is clear that the direction of movement in a negative reaction is not determined entirely by the position of the stimulating agent or the part of the body on which it acts. The moving amoeba is temporarily differentiated, having two ends of opposite character, while the two sides differ from the ends. These internal factors play a large part in determining the direction of movement; the present action of amoeba, even when responding to stimuli, depends, as a result of these temporary differentiations, partly on its past action. . . . In amoeba we see in the simplest way the effects of past stimuli and past reactions in determining present behavior (Jennings, '06, pp. 21 and 22).

These remarks of Jennings refer to weak or moderate stimulation. In regard to strong stimulation, Jennings makes the following statements:

But if all of one side or one end if strongly stimulated, then a pseudopodium may be sent out on the side opposite so that the animal turns almost directly away from the stimulated region (Jennings, '06, p. 6).

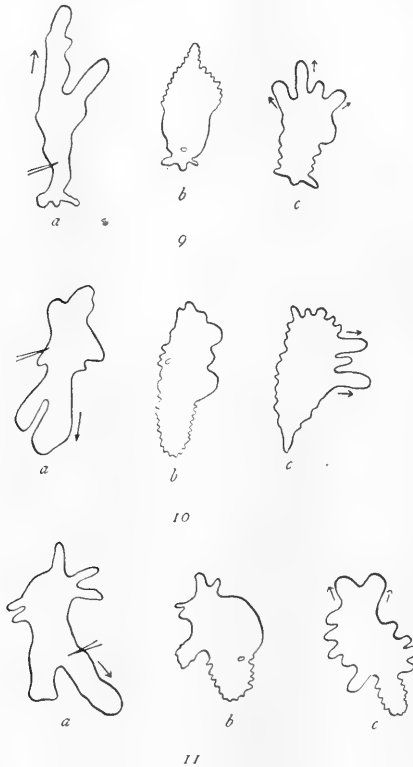
If the stimulus is very strong the contraction which takes place at the region stimulated may be very marked, resulting in the formation of strong folds in this region.<sup>8</sup> The contraction may include the entire anterior end of the Amoeba. Such a contraction destroys the attachment to the substratum, and the new pseudopodium now bursts out in some part of what was the posterior end of the body. The new course followed may then be at right angles to the old one, or at any greater angle or the course may be exactly reversed, the new pseudopodium being formed at the posterior end (Jennings, '04, p. 183).

<sup>8</sup> Jennings is here speaking of stimulation applied to the anterior end. He does not discuss the effect of strong stimulation when locally applied to other parts of the body.

The exposure of the amoeba to strong cyanide must be regarded as a strong stimulation; hence I have thought it advisable to study the reaction of the animal to other strong stimuli. I considered that mechanical stimulation would be the least objectionable and least complicated method of procedure, and therefore have employed as stimulating agent a needle point pressed against the animal with a force sufficient to completely pierce the protoplasm. Both the hay and wheat varieties of amoeba have been used.

When the amoeba is subjected to such mechanical stimulation, the immediate response is invariable. The stimulated region contracts more or less depending upon its previous degree of activity but the most striking effect occurs at the advancing end of the amoeba. This, whether it consist of one or more pseudopodia, contracts powerfully so that its surface is thrown into numerous short projections. The contraction of the anterior end, regardless of where the stimulus is applied indicates that conduction through the protoplasm is extremely rapid and effective. As a result of this contraction, the protoplasm flows towards the middle of the body which bulges out. (This heaping up of the protoplasm in the center of the body was also observed by Jennings, '04, p. 183.) After a little delay, new pseudopodia arise, and the animal moves away in evident haste. The place at which the new pseudopodia appear bears a direct relation to the place to which the stimulus was applied; it is opposite. In this regard strong stimulation differs from moderate or weak stimulation which, as Jennings says in the above citation, does not wholly determine the direction of subsequent locomotion. He has however observed that strong stimulation 'may' cause locomotion in the direction opposite to the point of application of the stimulus, and that strong stimulation applied at the anterior end practically always reverses the direction of locomotion. With this statement I am in complete accord, and to it I must add that when a strong stimulus is applied to any part of the body of the amoeba, the new pseudopodia appear opposite to the place of stimulation. I have experimented with a great many individuals, and have found this response to occur, not perhaps in-

variably, but certainly in the vast majority of cases. If the needle point is pressed against the anterior end, then the new pseudopodia appear at the posterior end; if applied at the posterior end, the anterior end first contracts, then resumes its advance, hastening its movement; if to one side, the opposite side, or the most distant end advances, and so on. I should state that I refer to the pseudopodia which appear first after the stimulation for these may not be the ones with which the animal



Figs. 9 to 11 Avoiding reaction of amoeba to strong mechanical stimulation. Figure 9a, stimulus applied to posterior end of an advancing amoeba; b, the anterior end contracts, and c, flows forward again.

Fig. 10a, stimulus applied to side of the amoeba; b, anterior end contracts; c, subsequent locomotion to side opposite that to which stimulus was applied. 11a, stimulus applied near anterior end; b, anterior end contracts; c, pseudopodia initiated at posterior end.

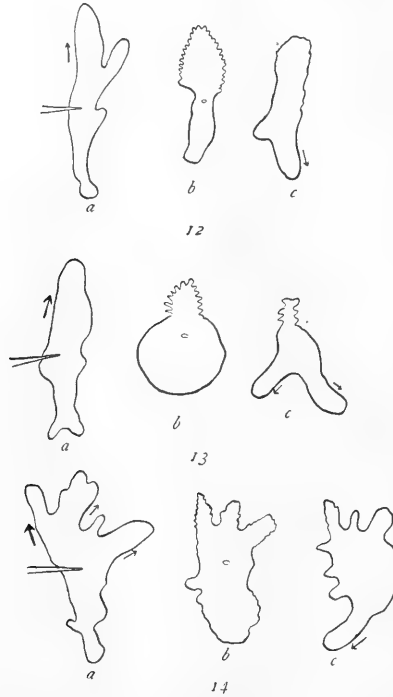
finally resumes locomotion. Figures 9 to 11 illustrate these reactions which may be regarded as avoiding reactions, in which the stimulus is completely directive.

Now it is necessary in order to imitate the action of the cyanide to use a non-directive stimulus, and I have attempted to accomplish this by thrusting the needle point through the center of the amoeba. The result of this procedure completely astonished me. I found that after the usual powerful contraction of the anterior end, the new pseudopodia almost invariably appear at the posterior end, or near the posterior end, that is, in the posterior half of the animal. I have repeated this experiment innumerable times, and in all cases where the stimulus is successfully applied to the center, the new pseudopodia have never been seen to arise from the anterior half but always from the posterior half of the amoeba. Figures 12 to 14 illustrate this reaction. The experiment is best performed on individuals with a single large pseudopodium (so-called limax type) since the needle can be more accurately placed. The hay amoeba (fig. 13) very frequently takes this form, while the wheat amoeba (figs. 12 and 14) tends to spread at its anterior end in a fan-like manner.

I have designated this reaction the shock reaction, as I conceive it to be a reaction to a strong but non-directive stimulus while the term avoiding implies that the localization of the stimulus has a directive effect on the response. Although it appears to be a fixed reaction, something like the reflexes of the higher forms, it is probably only a variation of the avoiding reaction, since Jennings has found that *Paramecium* when surrounded on all sides by a stimulating chemical repeatedly gives the avoiding reaction.<sup>9</sup> I suspect, however, that it would be more correct to say that the avoiding reaction is a variation of the shock reaction. The distinction, as I see it, between the two is one of degree only, namely, in the avoiding reaction, the

<sup>9</sup> I have found that amoeba gives the shock reaction when alcohol (about 50 per cent strength) is dropped upon it, and Mr. J. W. MacArthur of this laboratory has observed that the shock reaction is the initial effect of exposure of the amoeba to calcium chloride solution.

localization of the stimulus partially determines the direction of the response, and the internal conditions in the animal are also partially directive, while in the shock reaction, the stimulus is



Figs. 12 to 14 Shock reaction of amoeba. 12*a*, needle applied to center of the animal; *b*, anterior end contracts, and *c*, pseudopodia flow out from previous posterior end. 13*a*, the hay amoeba flowing with a single large pseudopodium; needle applied to center; *b*, anterior end contracts powerfully, causing rest of body to bulge; *c*, new pseudopodia appear at previous posterior end. 14*a*, the wheat amoeba showing characteristic fan-like advancing ends; needle applied to center; anterior half contracts in *b*, and new pseudopodium flows out posteriorly in *c*.

entirely non-directive, while the internal conditions wholly determine what shall be the direction of locomotion. The latter is, I should think, the more generalized type of response, from which a behavior resulting in an avoidance of the stimulating agent would be secondarily derived.

The shock reaction is readily capable of a purely mechanistic explanation. Since there exists in the moving amoeba a temporary metabolic gradient—and in other organisms a permanent gradient—a strong stimulus applied with equal intensity to all parts of the animal, and therefore not in itself differential, must nevertheless produce a differential effect, owing to the internal gradient. The most active end of the organism will be most affected by the stimulus and most inhibited, while the least active part is least altered and hence more capable of carrying out the normal activity. The anterior half is literally prevented from giving rise to pseudopodia.

These observations serve, I think, to remove any doubts as to the correctness of my interpretation of the initial rupture of the ectoplasm which occurs in normal cyanide. When the amoeba is exposed to cyanide of this strength, it gives the shock reaction. The contraction of the anterior end does not occur—this is probable impossible in the cyanide—but pseudopodium formation is initiated at the posterior end in the manner typical of the shock reaction. Whether pseudopodia actually appear or not depends upon the concentration of cyanide employed but in either event, the same internal change, increased susceptibility, takes place. From this it follows that this change, which I have interpreted as a metabolic change, is the direct cause of the formation of pseudopodia, and if long enough continued, of the pseudopodial gradient. The fact, then, that increased susceptibility, increased metabolic rate, precede pseudopodium formation constitutes a basis for certain suggestions regarding the nature of amoeboid movement.

#### IV. EXPLANATION OF AMOEBOID MOVEMENT

I wish to state at the outset that it is with considerable diffidence and a conviction of inadequacy that I enter upon a discussion of this difficult problem. I feel, however, that it is incumbent upon me to present the line of argument which naturally follows from my observations.

Anyone who writes upon the subject of amoeboid movement must give serious consideration to the surface tension theory because of the careful analysis to which this theory has been sub-

jected and the wealth of experimental data with which it has been presented. Other explanations of amoeboid movement have been so lacking in this respect that I shall not undertake to discuss them, with the exception of the contractility idea of Bellinger which will be referred to in the proper place.

The surface tension theory, as everyone knows, explains pseudopodium formation as the result of local diminutions of the tension of the surface of the protoplasm. This idea seems to have originated with Berthold ('86) who based it on certain well-known facts (due chiefly to the experiments of Quincke) concerning the spreading of fluids. Berthold's reasoning is as follows: if the sum of the surface tension between the amoeba and the water, and the amoeba and its substratum is less than the surface tension between the water and the substratum, then the amoeba will necessarily spread between the water and its substratum, owing to the tendency of any such system to bring its surface forces to a minimum value. In other words, anything that lowers the tension of the surfaces of contact of the amoeba will cause it to spread, and these spreadings will be local, i.e., pseudopodia, because of the impossibility of maintaining identical conditions on all sides of the animal. Berthold also explained the production of pseudopodia extending freely into the water on the assumptions that the tension between the amoeba and the water be reduced to zero, and that local differences exist in the water. Berthold, however, recognized clearly that alterations in the environment do not adequately explain amoeboid behavior but that chemical differences within the amoeba and modifications resulting from stimulation must be of great importance.

- This conception of Berthold's has been adopted by Rhumbler and has received extensive development in his hands ('98 and subsequent papers). Having made a careful study of the behavior of amoeboid organisms, Rhumbler attempted to imitate these activities with non-living materials, with such success that he concluded that both sets of phenomena are explicable by means of the same physical laws, the laws that govern the behavior of fluids, especially those concerning surface tension. Since, Rhumbler argued, inequalities in the surface tension will



cause fluid drops to change their shape and move about in amoeboid manner, such alterations of the surface tension due either to internal or external factors are the direct cause of the locomotion of amoeboid organisms.

Clearly this explanation of amoeboid motion is valid only if the protoplasm is fluid, for although the surface tension of solids is indeed very great, it plays no rôle in their behavior. It becomes incumbent, therefore, upon the supporters of a surface tension theory to demonstrate that the protoplasm is fluid. This Rhumbler has attempted in a number of papers ('98, '02, '03, and especially '14), where he endeavors to show experimentally that protoplasm is a fluid because it obeys the laws of fluids, namely, it is incompressible, inelastic, and exhibits the phenomena of capillarity. I do not wish to take the space here to criticize Rhumbler's argument adequately but it is certainly not convincing in many respects. My chief objections are: first, that he frequently uses material which can scarcely be regarded as representative protoplasm, as the yolk of avian and amphibian eggs, and, second, that many of his proofs concern the interior of the protoplasm (streaming movements, presence of spherical inclusions, etc.). That isolated pieces of protoplasm assume the spherical form is not necessarily a proof of its fluid condition, since the formation of a contractile membrane on the surface would explain the result (as Rhumbler himself remarks), and further the injury of cutting may completely alter the physical state of the protoplasm, probably in the direction of liquefaction.<sup>10</sup>

The surface tension theory plainly demands that the surface of the protoplasm shall be in the fluid state; the condition of the interior is of no moment. Now as a matter of fact when Rhumbler attempted to demonstrate that the surface of protoplasm obeys the laws of fluids he met with failure. An amoeba will not spread on the surface of water, as drops of yolk will, although if fluid it must do so, since the surface tension of protoplasm is less than that of water. If a fluid drop suspended in another fluid is

<sup>10</sup> Cf. Chambers ('17).

caused to rotate, its contents are also set in rotation, but if an amoeba or other living cell is rotated in the same way, its internal granules do not circulate. Dead protoplasm, however, exhibits the same phenomena as fluid drops. These discrepancies between the behavior of protoplasm and fluids can be explained only on the assumptions either that the surface of protoplasm is solid, or that it has the alveolar structure. In Rhumbler's own words ('14, pp. 513-514, 501-505):

Wenn des Amöben, als freilebende Plasmamassen durch ihren ganzen Leib hindurch flüssig wären, so wäre auch von ihnen die gleiche Ausbreitungserscheinung auf einer reinen Wasseroberfläche zu erwarten. Diese Erwartung bestätigt sich offenbar bei einer verschwindend kleinen Zahl von Amöben. . . . Bei einer grossen Überzahl der bis jetzt geprüften Amöben bleiben aber unter ähnlichen Umständen die Ausbreitungserscheinungen aus; es gibt sogar Amöben die nach Schneckenart auf der Unterseite der Wasseroberfläche hinkriechen können oder denen wenigstens eine direkte Berührung mit Luftblasen, die sie, sofern sie flüssig wären, auseinanderreißen müßten, gar nichts schadet. Dieses Resultat legt nahe, dass derartige Amöben zur Zeit der Ausführung des Experimentes keine flüssige, sondern eine als 'fest' zu bezeichnende Oberfläche besaßen. Auch die spitzen Pseudopodien von Aktinosphärium und die retikulosen der Foraminiferen vertragen die unmittelbare Berührung mit Luftschichten, ohne eine Ausbreitung der betreffenden Sarkodinen auf der Grenzfläche Wasser-Luft zu veranlassen . . . in den meisten Fällen ist die Oberflächenschicht der Amöben im Unterschied zu dem strömenden Innenplasma 'nicht flüssig,' sie ist in irgendwelcher Weise verfestigt; auch die Pseudopodien der Retikulosen ergeben sich durch die physikalische Probe, ebenso wie durch die empirische Beobachtung als nicht durch ihre ganze Masse hindurch flüssig, sondern als im Inneren mit gallertigen Achsenfäden ausgestattet . . .

Trotzdem aber wäre es falsch, aus dem nachgewiesene Streben der genannten Zelloberflächen nach Minimalflächen ohne weiteres ein Argument für den flüssigen Zustand der betreffenden Zelloberflächen ableiten zu wollen; es könnte sich vielmehr hierbei auch um elastisch gespannte Zellmembranen handeln; schon Zimmermann hat darauf hingewiesen, dass von einem Flüssigsein der Zelloberflächen überhaupt nur bei ganz jugendlichen Pflanzenzellen die Rede sein könne. . . . Es lässt sich sogar zeigen, dass die lebende Zelloberfläche niemals der Oberfläche einer einheitlichen leblosen Flüssigkeit mechanisch analog gesetzt werden darf, eine Tatsache die als "erste mechanische Inkongruenz zwischen leblosen Flüssigkeiten und lebenden Zelloberflächen" dadurch festgestellt werden kann, dass sich die Oberflächenteilchen einer einheitlichen Flüssigkeit innerhalb der Oberflächenschicht ebenso wie im Inneren ad libitum ohne sonstige Änderungen im System

mechanisch leicht verschieben lassen, während das in keinem Falle mit den Oberflächenteilchen der 'lebenden' Zelle geschehen kann. In ein flüssiges Medium eingesenkte, mit dem Medium nicht mischbare Flüssigkeitstropfen . . . . lassen sich durch genügend starke vorbeiziehende Strömungen . . . . stets in ihrem Inneren in konforme Strömungen versetzen, während das gleiche niemals bei einer lebenden Zelle gelingt. . . . . Verfährt man mit einer Amöbe in ähnlicher Weise, indem man Wasser über ihre Oberfläche hinspritzt, so wird man im Inneren der Amöbe keinerlei Strömungen ähnlicher Art wahrnehmen. . . . . Es fragt sich nun, ob die aufgedeckte Differenz etwa durch die Anwesenheit einer feinen, elastisch gespannten Zellhaut auf der Oberfläche der lebenden Zelle, auf die wir schon gemutet haben, erklärt werden kann. Sicher ist, dass eine derartige elastisch gespannte Oberflächenhaut bei den weitaus meisten Zellen in Gestalt der sogenannten Zellmembran deutlich vorhanden ist. Fraglich wird die Sachlage aber bei den sogenannten nackten, d. h. membranlosen Zellen, vor allem bei Amöben, amöboiden Zellen, und Furchungszellen. . . . . Beim Absterben derjenigen Zellen . . . . schlägt sich eine zähere Masse als flockiges Gerinnungsgerüstwerk innerhalb einer augenscheinlich sehr dünnen Flüssigkeit nieder. . . . . Wir sind gezwungen die zähere Masse als 'flüssige' innerhalb der weniger zähen, natürlich erst recht flüssigen verteilt zu denken. Es gibt aber nur eine Mischungsart von zwei 'Flüssigkeiten,' welche die Aufrechterhaltung der inneren Verschiebbarkeit die Oberfläche der Mischung soweit unverschiebbar macht, dass äusserlich vorbeigeführte Tangentialströme nicht analoge innere Rotationsströme in der Mischung erzeugen. Diese einzig mögliche Mischungsart ist die 'emulsoide Schaummischung,' oder um Bütschli's Ausdruck zu gebrauchen, die Wabenstruktur. . . . . für die ich . . . . die Bezeichnung 'spumoid' verwenden werde.<sup>11</sup>

We may now inquire a little more closely as to the meaning of this 'spumoid' structure. As is well known to all biologists, protoplasm is an emulsoid colloid in which the state of aggregation is readily altered by a variety of factors. In the sol state, the system is a fluid-fluid one, in which the disperse medium, or external phase, is water containing a great number of materials in solution and suspension, while the disperse phase consists of organic water-containing particles. In the passage from the sol to the gel state, according to most accounts, the system becomes a fluid-solid one, or at least one of the phases is nearly solid; the solid phase in dilute gels forms an open network, and in more concentrated gels becomes a honeycomb, that is, it constitutes the

<sup>11</sup> Italics of the original are omitted.

walls of polygonal cells in which the fluid phase is contained. (Bütschli, '94, p. 216; van Bemmelen, '98; Hardy, '99, '00; Freundlich, '09, p. 474; Bancroft, '13, '14; Clowes, '16a, '16b.)<sup>12</sup> If a gel has the reticular structure, neither phase can be said to be external since both are perfectly continuous; but in the alveolar structure the solid phase is external and continuous while the fluid phase is now internal and discontinuous, an exact reversal of the sol condition. The work of Bancroft and Clowes, just cited, in regard to such reversals is of great interest, especially as showing that no change of concentration of any of the gel-forming constituents, nor change of external conditions is necessary but only slight alterations of the chemical content. Clowes has very reasonably concluded that the interior of protoplasm is in the sol state with the colloidal proteins, etc., forming the disperse phase, while the surface layer of protoplasm is in the gel state with the fluid part dispersed, and the colloidal constituents forming solid walls and constituting the external phase.

These researches indicate that protoplasm when it passes into the gel state assumes the alveolar or spumoid structure, although of course we are not certain of this. If now, as Bütschli and Rhumbler state, protoplasm is alveolar or spumoid, or whenever or wherever it becomes so structured, one may reasonably conclude that it has passed into the gel condition, or at least that it has gained properties which are characteristic of solids and not of fluids. This is already evidenced by the behavior of foams, which consist of fluid walls inclosing gases but which exhibit properties not found in either gases or fluids but characteristic of solids. Solid properties must develop to a much greater extent in fluid-fluid or fluid-solid systems when they have an alveolar structure, in proportion to the viscosity of the external phase, which forms the walls of the alveoli. Thus in a viscous material

<sup>12</sup> Objections to this view are of course not wanting, e.g., Howell ('16). I am here merely presenting the view which is favored by the majority of investigators, and attempting to show that if emulsoid colloids have an alveolar structure in the gel state then the existence of alveolar structure in protoplasm (if it occurs) is indicative that such protoplasm is in the gel state.

like protoplasm, the assumption of the spumoid structure must be accompanied by a loss of fluid properties, and a marked acquisition of solid characteristics, such as compressibility, elasticity, contractility, etc.<sup>13</sup> Hence, Rhumbler's whole argument, the essentials of which are quoted above, that the discrepancies between the behavior of living protoplasm and of fluids offer two alternative explanations, i.e., the presence of a solid membrane, or the existence of the spumoid structure, really leaves him no alternative but leads on both sides to the conclusion that the surfaces of free cells do not behave like fluids but like solids. And from this it follows that amoeboid movement cannot be caused by alterations in the surface tension since surface tension phenomena as commonly understood are phenomena of fluids and the exposed surface of protoplasm behaves like a solid, and is in all probability a colloidal gel.

I now wish to present certain other lines of evidence which indicate conclusively that the surfaces of free cells, and particularly of amoeboid organisms are altogether too stiff and firm to obey the laws derived from the behavior of simple fluids. Reference may first be made to the micro-dissections of Kite ('13) and Chambers ('15, '17). Chambers states that with proper precautions no alteration of the physical condition of the protoplasm occurs as a result of the dissection. Kite found that the ectoplasm of the amoeba is a "quite concentrated gel while the interior is dilute," and that the "outer surface of *Amoeba proteus* is a semi-rigid solid of from 5 to 12 or more microns in thickness." The ectoplasm could easily be cut into pieces of all sizes. Chambers has confirmed these results and finds that free cells in general, such as egg cells and Protozoa consist of gel surfaces and sol interiors. The surface gel is contractile and extensible and if ruptured allows the cytoplasm to escape. A much more extensive rupture is required in the case of *Amoeba* than in the case of *Paramaecium* before the endoplasm will escape,

<sup>13</sup> Perhaps the most beautiful example of alveolar protoplasm is the protoplasm of the Heliozoa. I have found that the heliozoan *Actinosphaerium Eiehhornii* can be cut into pieces with a needle exactly as if it were solid, that is, the pieces show no tendency to minimize their surfaces. Obviously, the alveolar structure has conferred upon the protoplasm solid properties.

indicating the greater thickness of the surface gel in the former case. This I also have found to be true. I have frequently performed the following experiment on amoeba. The tips of the pseudopodia of the hay amoeba cling so firmly to the substratum that if a needle is applied to the posterior end and a pull exerted, the animals can often be pulled in two. In such cases the ectoplasm is stretched and drawn out into strands, a behavior that leaves no doubt that it is of a firm consistence and yet extensible.

Jennings ('04) records a great number of interesting observations that demonstrate the firm and contractile nature of the ectoplasm. For instance, in the retraction of pseudopodia either as a result of stimulation or in the course of locomotion, the surface of the pseudopodium is thrown into folds and corrugations. The posterior end is likewise always corrugated. I can fully confirm Jennings in these statements. This behavior necessitates the assumption, as Rhumbler ('05) recognizes, that the surface is heterogeneously solidified. Jennings also finds that the ectoplasm possesses elasticity and contractility, properties which again indicate its more or less solid character. The pseudopodia can be bent, as can the entire body, and both return to their original position, a property of solids, not of fluids. The ectoplasm acts like a 'tough skin.' When a contracted pseudopodium, composed of ectoplasm alone again comes into action, as often happens, the returning endoplasm straightens it out "with a sort of jerk," as if it were an empty wrinkled glove finger. I have frequently made the same observations. Dellinger ('06) observes that amoebae may be rolled about with a needle; they not only retain their form under such treatment but the endoplasmic granules do not shift their positions. This again indicates a degree of firmness and solidity of the ectoplasm which is incompatible with the surface tension theory, and shows also that the endoplasm is not very fluid, or at least that it may become fairly rigid.

It seems unnecessary to recount here any further observational details which demonstrate the non-fluidity of the ectoplasm. I have made a great many such observations myself but found

later that most of them had already been recorded by Jennings in his 1904 publication to which the reader is referred. It will be sufficient for me to mention that the method of disintegration of the amoeba in cyanide, as described earlier in this paper, points to the same conclusion; and to report an interesting case of an ingestion of a rotifer by an amoeba. The amoeba had assumed a nearly spherical form following its capture of the rotifer; the rotifer, which appeared to be quite intact, was thrashing about vigorously within the amoeba, doubling and straightening itself. The endoplasm offered no obstacle whatever to the frantic movements of the rotifer but all its struggles did not enable it to pierce the ectoplasm or to produce any effect upon it. The ectoplasm offered a seemingly solid barrier to its escape, behaving indeed like a 'skin' containing the much more fluid endoplasm.

Another line of evidence is afforded by those Rhizopoda which habitually produce permanent long and slender pseudopodia, as the Foraminifera and the Radiolaria. Even *Amoeba* may give rise to quite slender pseudopodia. I find it impossible to understand how a fluid could be drawn out into such long processes without breaking, and, how, if this were possible, it could maintain these processes against the surface forces. As Rhumbler has quite readily admitted, one is compelled to recognize that the protoplasm of these organisms is to some degree solidified, as the presence of a visibly solid axis in many cases testifies. Perhaps the most remarkable case of this kind is that of the Foraminiferan *Astrorhiza*, described by Schultz ('15), whose long thread-like pseudopodia, after becoming fastened at their tips, exhibit tensile and contractile properties very much like those of stretched rubber bands; if cut, they retract instantly, just like stretched rubber, and they contract normally with sufficient force to draw the animal along.

The actual method of locomotion in amoebae and related forms furnishes further excellent evidence against the correctness of the assumptions of the surface tension theory. *Amoeba* does not move in complete contact with the substratum as supposed by Berthold, Rhumbler and others, nor in the rolling manner described by Jennings ('04), except in the case of *Amoeba verru-*

cosa, and possibly some other species. As Dellinger ('06) first showed, *Amoeba proteus* and other rhizopods are attached only at limited points, the tips of the pseudopodia, and generally the posterior end also. Pseudopodia are first extended free into the water, then attach at their tips with more or less curvature, then contract. The amoeba 'walks' on the tips of its pseudopodia. This behavior seems to me completely incompatible with the idea that the amoeba is fluid and compels the conclusion that the ectoplasm is more or less rigid. I may say that there is no doubt that both of the varieties of *Amoeba proteus* which I have used move exactly in the way described by Dellinger. This is readily determined by careful focusing, by passing a needle point under the animal, etc. without the use of the horizontal method of observation. I have frequently observed the animals 'walking' on the under side of a cover glass with the tips of their pseudopodia flattened against the glass, while the central mass hung free in the water. The tips of the pseudopodia cling to the substratum with such force that strong squirts from a pipette are required to detach them, and the amoebae may be pulled in two, as described above, before they will loosen their hold. If a moving amoeba is quickly detached, and rolled about with a needle, the form, shape and curvature of the pseudopodia can easily be determined, for these are retained for some time, and one can often even see the flattened plane surface of the previously attached pseudopodial tip, which persists for a little while in this condition in defiance of all the laws of minimum surface! Anyone who makes these observations can have no particle of doubt regarding the existence of solid properties in the ectoplasm.

I believe, however, that Dellinger's discovery while practically proving the contractile nature of the ectoplasm, though no more so than do Jennings' numerous and careful observations, does not justify his assumption that a 'contractile substance' is present in the ectoplasm, nor his comparison of amoeboid motion with that of an animal like the leech which is provided with contractile fibers. As Rhumbler in his reference to Dellinger ('07) has justly remarked, nothing is gained by referring amoeboid motion to contractile elements, since what we are trying to do is to explain



the physical chemistry of contractility by observing and experimenting with amoeboid motion, its simplest expression. Rhumbler has also conclusively shown the indefensibility of Dellinger's point of view, in that amoeboid movement must be due to alterations of the surface of some kind, for the simple reason that the surface actually does change, this being the most obvious feature of the amoeboid kind of movement.

Another objection to the surface tension theory is that based on the endoplasmic currents. These do not agree with the currents produced in drops of fluid by local diminutions of the surface tension. In such drops, the current flows forward in the center towards the area of decreased tension, and away from it in the entire surface. These currents are called 'fountain currents' by Rhumbler, and he thought the endoplasmic currents in various Rhizopoda were identical with these fountain streams. No one would presume to doubt the validity of Rhumbler's observations on the currents in *Amoeba blattae*, *Pelomyxa*, and other forms as reported in his papers of '98 and '05, but the fact remains that in most amoebae, and certainly in *Amoeba proteus*, there is no backward flowing of the surface layer. This was first pointed out by Jennings ('04) who remarks that Wallich had noticed it in 1863, and Jennings has given a full discussion of the matter to which I may refer without further comment. Dellinger also notes the absence of any backward flowing of the granules, and I myself must agree completely with the account of Jennings (*loc. cit.*, p. 135). In fact, according to the observations of Jennings, Dellinger, Gruber ('12), and Schaeffer ('16),<sup>14</sup> the surface of the ectoplasm actually flows forward at about the same rate as the forward advance, and this indicates that the advancing ectoplasm at the tip of the pseudopodium is derived from the surface ectoplasm and not from a transformation of endoplasm into ectoplasm at the end of the pseudopodium as Rhumbler supposed. Rhumbler in his discussion of Jennings' work (Rhumbler, '05) says that the absence of the backward currents is the result of the gelation of the surface, leading therefore to

<sup>14</sup> The forward movement of the surface layer of amoeba seems to have been noticed first by Bütschli ('94, p. 328).

the same conclusion that we have reached from other lines of evidence.

Finally I may mention the observations of Mast and Root ('16) on the feeding of *Amoeba* on *Paramecium*. They frequently saw an amoeba partially inclose a *Paramecium* with food cup, and then cut the *Paramecium* in two by bringing the edges of the cup together. They calculated that the force necessary to sever a *Paramecium* in this way is very much greater than any which could possibly be developed through differences between the surface tension of the rim of the food cup and the surface tension elsewhere. The argument is obviously open to the criticism that the amoeba may extrude something which softens the surface gel of the *Paramecium* but this appears to be ruled out by the rapidity with which the process occurs—10 seconds—as well as by the consideration that such softening would probably cause visible changes in the *Paramecium* which could scarcely have been overlooked by the observers.

Probably a great many more facts of experiment and observation could be brought forward as evidences against the surface tension theory. I have not attempted a thorough search through the literature. I believe that the facts which I have already presented are quite sufficient to convince one that the ectoplasm of the amoeba is solidified to a degree which makes it highly improbable if not impossible that surface tension can play any important rôle in amoeboid movement, and further that the behavior of amoeba in general is incompatible with such an idea.

We may therefore come to the following conclusions regarding the physical state of the protoplasm of the amoeba and probably many other Rhizopods. The ectoplasm is an emulsoid gel, semi-rigid and more or less solidified. Like all such emulsoid gels, it possesses many properties of solids, such as high elasticity, contractility, extreme viscosity, compressibility (cf. Freundlich '09, pp. 474 ff.). These properties are in all probability due to the structure of such gels; they are probably made up of polygonal cells, the thin walls of which consist of the colloidal ma-

terial, proteins, carbohydrates, and lipins, while the cavities of the cells are filled with fluid. This structure is identical with the alveolar and spumoid structure of Rhumbler and Bütschli so that any conclusions or theories which they have based on this structure must ipso facto be referred to the gel condition.<sup>15</sup> The endoplasm is a viscous sol, whose degree of fluidity is capable of considerable variation. It is probable that the ectoplasm and the endoplasm grade into each other imperceptibly. I am much in favor of Clowes' interpretation, already referred to, that in passing from the surface of the protoplasm to the interior, a reversal of phase occurs, the colloidal material forming the outer phase, or disperse medium, in the surface layers, while in the interior it forms the disperse phase and water containing a variety of materials in solution and suspension, is the disperse medium.

It is very probable that in amoeboid organisms the surface layer will be found to show all possible gradations from real fluidity to extreme solidity and rigidity. The *Amoeba blattae* referred to by Rhumbler is undoubtedly one of the more fluid forms, while *Amoeba verrucosa* has a highly rigid ectoplasm.<sup>16</sup> Different degrees of surface solidity appear to constitute the chief differences between the two varieties of *Amoeba proteus* which I have employed. The variety grown in hay culture has the more rigid ectoplasm; its pseudopodia are more definitely localized, are often very long and slender, do not tend to fuse, and are very firmly fastened at their tips to the substratum. The variety grown in wheat has broader illly-defined pseudopodia, which are seldom long and slender but tend to fuse sideways so as to give a fan-like advancing end; the animal is not very firmly fastened to the substratum, although more of its surface is in contact with it. Owing to its greater rigidity, the hay variety shows the disintegration gradient much more clearly than the wheat variety.

<sup>15</sup> I wish again to point out that the evidence for this structure of emulsoid gels is not as yet conclusive but if one assumes it to exist in protoplasm one must realize the loss of fluid properties which its presence entails.

<sup>16</sup> *Amoeba terricola* is said to be covered by a definite resistant membrane (Grosse-Allermann, '09).

• Without then any intention of denying that amoebae with fluid surfaces exist, I much nevertheless insist on the basis of the facts presented above that in the great majority of the Rhizopoda, the ectoplasm has undergone gelation; indeed, this would seem to be the necessary consequence of the exposure of such a colloidal solution as protoplasm to the water. Thus it has frequently been demonstrated that isolated portions of cells quickly form the same sort of surface 'membrane' as was present on the original cell. It is unnecessary to point out that fluid protoplasm in the absence of such a surface layer would quickly diffuse out into the water, as happens in the amoeba when the ectoplasm is destroyed.

I may now state an explanation of amoeboid movement based on the foregoing facts. Since the ectoplasm is a more or less rigid gel, the direct cause of pseudopod formation must be a local liquefaction, and the direct cause of the withdrawal and contraction of pseudopodia must be coagulation. Thus gelation and solation<sup>17</sup> are the essential processes in amoeboid motion. The metabolic change which I have shown with the cyanide method to precede pseudopod formation is undoubtedly the cause of the solation or liquefaction; the cause of the gelation is not so clear, but it must be a reversal of the changes which bring about liquefaction.

According to this idea, an amoeba moves in the following way. The ectoplasm is an elastic tensile gel which exerts a tension upon the more fluid endoplasm. This elastic tension—the 'gelatinierungsdruck' of Rhumbler—is characteristic of colloidal gels and appears to be very much greater than the surface tension

<sup>17</sup> I have used the terms gelation and solation in preference to the more awkward but perhaps more familiar words gelatinization and peptonization. By gelation I mean an increase in size of the colloidal particles due to aggregation, resulting in a great increase in viscosity, and by solation, a decrease in size of the particles, a finer state of dispersion, with decrease in viscosity. I have avoided a discussion of the relation between viscosity and colloidal state because this subject is in a very unsatisfactory state at the present time. I have also avoided a discussion of the energy changes involved in such alterations of the colloidal state but obviously they must be very great. I prefer not to use the term coagulation because this generally implies a separation out of the colloidal material, an occurrence which seems to be incompatible with life.

characteristic of fluids. At some local spot in this gel, a chemical change occurs, an increase in metabolic rate or an alteration of some kind of metabolism. As a result of this, substances or ions are set free or removed which alter the colloidal state of the ectoplasm, and cause it to become more liquid, that is, to go over into the sol state. If emulsoid gels have an alveolar structure,<sup>18</sup> this would involve dissolution of the walls of the alveoli and a reversal of phase such as Clowes has suggested. These reversals are easily and rapidly brought about by the addition of the proper ions. At the region where the liquefaction has occurred, the protoplasm must necessarily flow out owing to the tension exerted by the ectoplasm. As soon, however, as the pseudopodium extends out into the water, its surface gelatinizes because of contact with the water. It is necessary therefore for the continuous production of a pseudopodium, that the metabolic change which is the cause of the liquefaction should continue to occur at the pseudopodial tip. There is thus produced the metabolic gradient along the pseudopodium which I have described in the earlier part of this paper. The pseudopodium thus coagulates as it goes so that it may readily curve or bend or assume and maintain any of the irregular forms often seen, and further it is sufficiently stiff to support the amoeba in the way that Dellinger discovered.

While I have based the idea that pseudopod formation is the result of liquefaction mainly on logical grounds, direct observational evidence to the same effect is not wanting. Thus many observers have noted that the newest pseudopodia are much more fluid than the older ones, and that pseudopodia which have been long in contact with the water are quite stiff and rigid and are dragged about by the amoeba like inert masses (for instance, Gruber, '11). Observation of the amplitude of the Brownian movement of the endoplasmic granules confirms these statements. While it is indeed somewhat difficult to compare the Brownian movements of the granules in various parts of the amoeba with any degree of certainty, yet I am reasonably sure

<sup>18</sup> The existence of the alveolar structure is not of course in the least necessary for the theory presented here.

that the Brownian movement is greater in the more internal than the superficial granules, and in the tips of the pseudopodia, during or just after the cessation of locomotion, than elsewhere in the ectoplasm. Other evidence is furnished by careful observation of the locomotion under high power. The pseudopodium advances by little lunges, first to one side, then to the other, just as it should if sudden local liquefaction were occurring.<sup>19</sup> This method of advance is more marked, as it should be, in all cases where the ectoplasm is more gelatinized, as when movement starts up in a pseudopodium which has been abandoned for a little time, or when the amoeba starts to move from the contracted condition, etc. It is also far more obvious in the hay variety than in the wheat variety, and the former, as I have given reason for thinking, has the more rigid ectoplasm. The 'eruptive' pseudopodia of Rhumbler and others are the same lunges of the ectoplasm on a larger scale, and appear to be characteristic of amoebae with highly gelatinized ectoplasms since the ectoplasm which had previously formed the surface remains visible for some time within the erupted pseudopodium, and forms a barrier to the advance of the endoplasm. These ectoplasmic barriers are always observable in the amoebae I have examined, particularly in the hay variety.

I have stated that the withdrawal and contraction of pseudopodia are processes of gelation. This, I think, follows inevitably if the opposite process is one of solation. The chief other ground for it is that contraction is always accompanied by the appearance of folds and corrugations in the ectoplasm, which are the more marked the more sudden and powerful the contraction.

<sup>19</sup> It would seem to be a simple matter to determine in what part of the pseudopodium movement is initiated, but as a matter of fact it is very difficult. Harrington and Leaming ('00) state that movement begins in a mass of granules in the middle of the pseudopodium but I am unable to agree with this. I find the movement starting in the clear non-granular ectoplasm, which frequently lunges forward without any corresponding movement in the endoplasmic granules. I am not prepared to explain the forward movement of the surface of amoeba, which has been described by several investigators, most recently Schaeffer ('16), since the various accounts do not agree. I am inclined to agree with Schaeffer that it indicates that the ectoplasm at the tip of the pseudopodium increases at the expense of the surface ectoplasm and not at the expense of the endoplasm.

The most reasonable explanation of this occurrence is, as Rhumbler has pointed out in an excellent discussion ('05, pp. 29 ff.), heterogeneous tension in a gelatinized surface, these differences in the tension being due, of course, to differences in the degree of gelation. One may say then that contraction in the amoeba is the result of differential gelation. I do not undertake to say why protoplasm undergoing gelation should develop the property of contractility. The fact remains that emulsoid solutions in general when they pass into the gel state do develop this property, and I am quite content to leave the explanation to the colloid chemists. Mathews in his *Physiological Chemistry* (2nd edition, '16, pp. 232-233) has given an explanation.

I should therefore state emphatically that the property of protoplasm which we call contractility is nothing more or less than the gelation of an emulsoid colloidal solution; or, as we like to say in science, gelation is the 'cause' of contractility.

An observation made by Gruber ('12, p. 326 ff.) is of interest as further proof that the folding of the ectoplasm in contraction is due to coagulation. The normal contracted amoeba is a spherical mass studded with short pseudopodia-like projections. If now the temperature be raised (30°C. in Gruber's experiment), these projections disappear and the surface is smooth, and obviously highly fluid. On lowering the temperature, the surface again becomes corrugated. I have also frequently observed that when the cultural conditions become unfavorable, the amoebae cease to move, and take on a spherical form with a smooth surface. That such amoebae have become fluid is indicated by the very marked Brownian movement of their granules, as well as by the great increase in the size of the contractile vacuole (also noted by Gruber at high temperature) and in the amount of fluid in the food vacuoles. One may say that in the amoeba a smooth surface indicates an alteration of the colloid state toward fluidity while a corrugated and folded surface indicates alteration toward coagulation.

The liquefaction, as I have already pointed out, is caused by chemical changes in the amoeba involving probably the setting free of substances which produce liquefaction or the removal

of substances which induce coagulation; the coagulation is presumably the reverse bringing about the removal or recombination of the liquefying substances or production of coagulating substances.<sup>20</sup> On this point, however, I have no evidence. These changes which are the causes of amoeboid movement and behavior originate within the amoeba, and external stimuli do not act directly to produce those physical alterations which result in movement but they act through the protoplasm of the amoeba. I consider that the reactions of amoeba are similar in kind to the reflexes of higher forms, involving reception of the stimulus, conduction, and the initiation of changes leading to a response, except that here of course, receptor, conductor and effector are not differentiated. I should not at all deny that an external change could bring about directly liquefaction or coagulation and resulting locomotion but I think it highly improbable that this occurs under the usual conditions of existence of the amoeba.<sup>21</sup>

I am naturally quite unable to explain how the amoeba alters its own metabolic processes, or how it is determined where the local liquefaction in the ectoplasm is to occur. One becomes involved in the old question as to whether living matter is ever able to originate a change or whether everything it does is the result in the final analysis of changes in the external world. If the first supposition is true that protoplasm is capable of self-

<sup>20</sup> The contraction of the amoeba corresponds to the contraction of muscle, and the putting forth of pseudopodia to the relaxation of muscle. If there is any similarity between the two, contraction in the amoeba may be due to the production of some substance, since contraction of muscle is accompanied by the appearance of lactic acid. On the other hand, the putting forth of pseudopodia may be the result of the removal of this substance, as in the relaxation of muscle, where the lactic acid disappears, and this disappearance is accompanied by and seems to be dependent on increased oxidation processes. This again is similar to the amoeba, where pseudopodium formation is preceded by increased metabolism. I am very doubtful, however, that there is any resemblance in detail between amoeboid movement and muscle contraction, although there very probably is a resemblance in a large way (*infra*).

<sup>21</sup> Greeley ('04) found that cations coagulate the protoplasm of Protozoa, and that they cause contraction in the amoeba, while anions liquefy the protoplasm, and similarly anions bring about the extension of pseudopodia in amoeba.



stimulation, then we must add to the list of properties of living matter a sort of psychic property which would be possessed by the amoeba, and possibly by all matter (cf. Mathews, '16, p. 595). If on the other hand, one denies to protoplasm, even to the thinking cells in the cerebral cortex of man, the capacity for self-stimulation, or automaticity, then one must refer the activities of the amoeba to present or past conditions in its environment, and to internal conditions evoked by environmental changes. In this connection it is interesting to note the great tendency of the amoeba to bring into action again pseudopodia which had been abandoned rather than to form new ones,—an illustration of the effect of past conditions in determining present behavior.

A number of researches demonstrate that the nucleus plays an important rôle in amoeboid movement. It has been generally found that non-nucleated pieces of amoeba are incapable of normal amoeboid locomotion; they assume a spherical form, and undergo slight changes of shape with the formation of short broad pseudopodia but appear to be unable to form long pseudopodia or to attach them in the usual way. Details of the behavior with references to earlier work will be found in Willis ('16), with whose account I must agree completely. Mr Vernon Lynch, of Johns Hopkins University, kindly allows me to report that he finds that non-nucleated pieces are generally more susceptible to cyanide than nucleated ones, and always so if they formed the anterior end of the amoeba at the time of cutting (pieces tested one hour after cutting). It would be premature to attempt to explain the rôle of the nucleus without further experimentation, but I might suggest that the nucleus is concerned in the chemical processes which underlie the alterations of physical state necessary for locomotion.

After I had developed the theory of amoeboid motion which has been outlined in the preceding pages, I found that R. S. Lillie had given a similar explanation for muscle contraction ('06, '08, '12). He believes that the muscle fibril is a gel, the anisotropic segments being more concentrated than the isotropic

ones,<sup>22</sup> and that the direct mechanical cause of the contraction of the muscle is the coagulation in the anisotropic segments with displacement of fluid into the isotropic ones. The relaxation is the reverse process. Lillie thinks the cause of the coagulation is the depolarization of the cell membranes due to the nerve impulse. In the amoeba, of course, there is no nerve impulse, no constant external factor to initiate the chemical changes which precede locomotion, and certainly one cannot postulate a depolarization of the surface. It is indeed a far cry from the locomotion of an amoeba to the contraction of a striated muscle but we may perhaps believe that a similar principle is involved in both, although doubtless working in a very different way and through a different structural mechanism. We may imagine that nature starting with a colloidal solution such as protoplasm has been able to make it move by the very simple method of alteration of the colloidal state, and then gradually evolved a structure which could use the same principle in an infinitely more effective way.<sup>23</sup>

Lillie (*loc. cit.*) has referred to one case in which contraction is accompanied by visible coagulation, namely, the ctenophore swimming plate. The contractile activity of this structure, if artificially accelerated, is accompanied by a progressive coagulation of its substance. Various similar facts of coagulation accompanying contraction are known for vertebrate muscle, but their interpretation is not as clear as in this case. Another clear-cut case is that of the foraminiferan *Astrorhiza* (Schultz, '15). The surface of this animal consists of a sticky 'skin' which can be drawn out into long threads. The inner protoplasm is quite different, and cannot form threads. The organism normally puts out very long slender pseudopodia in bundles. The ends of these move about in the water and finally attach themselves. After this attachment, the pseudopodia develop a very

<sup>22</sup> This statement finds complete confirmation in the dissections of Kite ('13), who states that living striated muscle is the most "viscous, elastic, and cohesive of living gels," and that the dark segments are more concentrated gels than the light segments.

<sup>23</sup> I consider that it would be futile to attempt to discover the details of the mechanism of striated muscle by studying amoeboid movement.

great elastic tension, so that if they are cut they retract and shorten like stretched rubber bands when cut. With the development of this tension there appears in each pseudopodium, an axial fibril, obviously indicative of coagulation. The animal moves by the contraction of the pseudopodia, drawing itself along, and in this contraction, the axial fibril shortens and does not wrinkle, indicating that it is the contractile element. When the pseudopodia are withdrawn the fibrils vanish completely. This appears to me to be excellent case of coagulation accompanying contractility.

In conclusion, I wish emphatically to disclaim any originality or priority for the explanation of amoeboid movement which I have presented, although I should perhaps state that it arose independently in my mind as a result of my observations on the axial gradient. I found in the literature that a number of investigators had made similar suggestions, particularly Jennings,<sup>24</sup> and further that Rhumbler as far back as 1898 had given the same explanation of the movement of certain amoebae with 'hautartigem' ectoplasm, such as *A. verrucosa*. He states that in amoebae with solidified ectoplasms "die Bewegung des Pseudopodiums erfolgt dadurch dass lokale Verflüssigungen bez. Herabminderungen der Spannkraft der Haut . . . herbeigeführt werden" (Rhumbler, '98, p. 195). In later papers ('05, '10) he has given a more complete statement of the method of locomotion in amoebae with gelatinized surfaces, and in various places he has discussed the futility of attempting to draw any line between the liquid and solid state in emulsoid solutions, and has emphasized that in protoplasm the state is always changing. I believe that Rhumbler has been mistaken merely in assuming that most amoeboid organisms have fluid surfaces, and in failing to realize that the spumoid structure is evidence of the loss of fluid properties and the gain of solid properties. The 'Schaumspannung' to which he frequently refers is identical with the contractile tension on the surface of amoeboid organisms of which I have spoken. It is a matter of

<sup>24</sup> Even Bütschli had a very similar theory ('94, p. 314); also Montgomery (Pflüger's Archiv, vol. 25, 1881).

surprise to me that Rhumbler's explanation of amoeboid movement in forms with gelatinized surfaces has not received wider recognition. In this paper, I have only attempted to restate his theory in the light of my observations on the presence of the pseudopodial gradients, which necessitate the conclusion that the mechanism of amoeboid movement is internal.

#### SUMMARY

1. A gradient in susceptibility to potassium cyanide exists in each pseudopodium of amoeba. The susceptibility is greatest at the distal end and decreases proximally. The most recent or most recent vigorous pseudopodia are the most susceptible.

2. An amoeba is therefore temporarily differentiated into anterior and posterior ends, the region or regions of highest susceptibility being the anterior end or ends as in other organisms.

3. It is shown that the susceptibility gradient, which is a metabolic gradient, arises in the amoeba before the pseudopodium appears and hence the metabolic change which produces increased susceptibility is the primary cause of pseudopodium formation.

4. Evidence is adduced to show that the surface of most amoeboid organisms is in a state of gelation

5. From 3 and 4, it is argued that amoeboid movement must be due to alterations of the colloidal state. Liquefaction or solation is regarded as the cause of the extension of a pseudopodium, and coagulation or gelation of the withdrawal of pseudopodia and of active contraction. The liquefaction is believed to be brought about by the metabolic change referred to in 3.

6. Attention is directed to the fact that this theory is only an extension of one previously advanced by Rhumbler.

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## THE RELATIVE INFLUENCE OF FOOD AND OXYGEN IN CONTROLLING SEX IN ROTIFERS<sup>1</sup>

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FOUR FIGURES AND FOUR DIAGRAMS

It has been shown in a former paper that in five different species of rotifers the production of male-producing females and female-producing females can be regulated at will by certain manipulations of the food supplies. In some species a continuous diet of a colorless flagellate, *Polytoma*, caused only female producing females to be produced but when a green flagellate, *Chlamydomonas*, was substituted for the colorless flagellated food nearly all male-producing females were produced. In one species, the only one upon which such experiments were made, a scanty diet of pure cultures of the green flagellate, *Chlamydomonas*, caused all or nearly all female-producing females to be produced while a more abundant diet of the same pure cultures of *Chlamydomonas* caused as high as 90 per cent of male-producing females to be produced.

Shull and Ladoff working with *Hydatina senta* have since suggested and indicated in the results of their experiments that an excess of oxygen in the culture water may cause a greater production of male-producing females than in experiments without an excess of oxygen.

Since the publication of the former paper experiments have been carried on with four additional species of rotifers which also showed that a scanty diet of food caused female-producing females to be produced while a copious diet caused male-producing females to be produced. These species used were identified by Harry K. Haring, Custodian of the Rotatoria in the United States National Museum, to whom I am greatly indebted.

<sup>1</sup> Studies from the Zoological Laboratory, The University of Nebraska, No. 118.

There has also been carried on quite an extended series of varied experiments with several species of rotifers to determine the value of oxygen as a factor in causing male-producing females to appear. The experiments with the four additional species of rotifers concerning the production of male-producing females and female-producing females by increasing or decreasing the food supply will be presented first and following these there will be presented the experiments with oxygen. The method of making the stock bouillon, the stock stable tea, and of rearing pure cultures of *Chlamydomonas* have already been previously described in earlier papers.

#### BRACHIONUS MILITARIS

On January 7 of 1916 four liters of water and a small amount of sediment were collected from a small pond near Middletown, Connecticut. This collection was put into a battery jar which was placed in a large pan of running tap water in a south window; thus allowing the jar to be in sunlight but at the same time preventing any considerable rise in temperature by means of the running water. A small amount of the stock stable tea was added to the jar and then it was allowed to stand undisturbed during the remainder of January. During February small quantities of *Chlamydomonas* were added to the jar and on March 1 there were many thousands of females of *Brachionus militaris* all of which were carrying female eggs. During the following five days considerable quantities of the *Chlamydomonas* were added daily to the jar and on March 6 about one-half of the many thousands of females were carrying female eggs and the other half were carrying male eggs. During the following five days no *Chlamydomonas* was added and on March 11 about 90 per cent of the many thousands of females were carrying female eggs and about 10 per cent were carrying male eggs. During the following four days considerable quantities of the *Chlamydomonas* were again added and on March 15 about one-fourth of the many thousands of females were carrying female eggs and the other three-fourths were carrying male eggs. The complete data of these experiments will be found in table 1, a plotting of

TABLE 1

*Mass culture experiment with Brachionus militaris showing that when considerable quantities of the green flagellate, Chlamydomonas, were added to the jar of water that many male-producing females appeared but when only small quantities of Chlamydomonas or none at all were added very few, if any, male-producing females appeared*

TIME 1916	FOOD CONDITIONS	ESTIMATED NUMBER OF FEMALES OBSERVED		
		♀ ♀	♂ ♀	Per cent of ♂ ♀
January 7	4 liter battery jar of pond water collected. Stable tea added			
January 7-31	Miscellaneous protozoa growing in jar			
February-March	Small quantities of Chlamydomonas added			
March 1		500	0	0
March 1-6	Considerable quantities of Chlamydomonas added daily			
March 6		500	500	50
March 6-11	No Chlamydomonas added			
March 11		900	100	10
March 11-15	Considerable quantities of Chlamydomonas added daily			
March 15		250	750	75

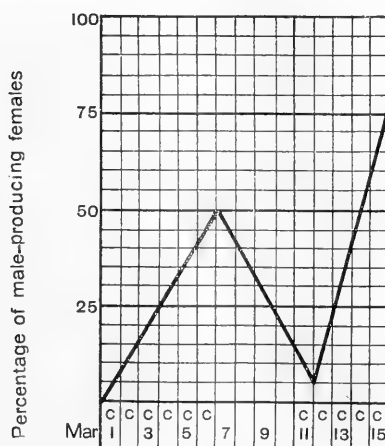


Diagram 1 *Brachionus militaris*. Experiment of table 1. Showing the production of a high percentage of male-producing females when the food conditions were changed by the additions of considerable quantities of the green flagellate, *Chlamydomonas*. C indicates the addition of the *Chlamydomonas*.

the results in diagram I, and drawings of the females showing the different eggs attached and also of a male will be found in figure 1.

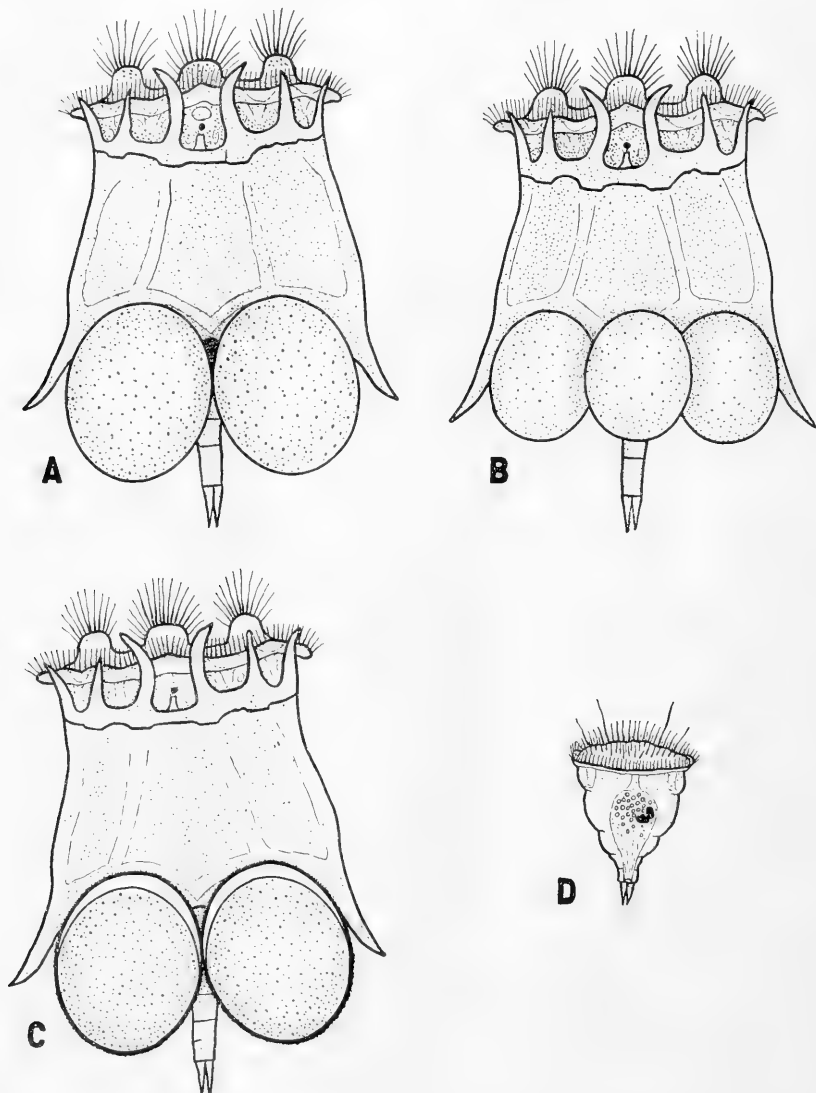


Fig. 1 *Brachionus militaris* (dorsal views). *A*, female with attached parthenogenetic female eggs; *B*, female with attached parthenogenetic male eggs; *C*, female with attached fertilized eggs; *D*, male.

## BRACHIONUS BAKERI

During the spring of 1916 several collections of water and sediment were made from the ponds in the vicinity of Middletown, Connecticut. The rotifer, *Brachionus bakeri*, was very frequently found in these collections. Individual isolation ex-

TABLE 2

*Mass culture experiments with Brachionus bakeri showing that when considerable quantities of the green flagellate, Chlamydomonas, were added to the jar of water that many male-producing females appeared but when only small quantities of Chlamydomonas or none at all were added very few if any male-producing females appeared*

EXPERIMENT	TIME 1916	FOOD CONDITIONS	ESTIMATED NUMBER OF FEMALES OBSERVED		
			♀ ♀	♂ ♀	Per cent of ♂ ♀
1	March 15	4 liter battery jar of pond water collected			
	March 15-May 9	Small quantities of <i>Chlamydomonas</i> added occasionally			
	May 9		98	2	2
	May 9-16	Considerable quantities of <i>Chlamydomonas</i> added at 48 hour intervals			
	May 16		130	70	35
	May 17	Considerable quantity of <i>Chlamydomonas</i> added	80	120	60
	May 18	Considerable quantity of <i>Chlamydomonas</i> added	75	225	75
	May 20		60	240	80
2	May 25	4 liter battery jar of pond water collected	49	1	2
	May 25-June 2	Considerable quantities of <i>Chlamydomonas</i> added at 48 hour intervals			
	June 2		150	350	70
3	May 25	Same conditions as in experiment 2	49	1	2
	June 2		105	195	65
4	May 25	Same conditions as in experiment 2	49	1	2
	June 5		100	900	90

periments were attempted but proving too tedious because of various difficulties in the technique of feeding and isolation they were abandoned and mass cultures were reared in four liter battery jars. These jars were placed in a large pan of running water in a south window and soon various kinds of protozoa developed in them. Small quantities of *Chlamydomonas* were added occasionally until the jars were well balanced and several thousand of the rotifers had developed. At this period nearly all of the females were carrying female eggs. Considerable

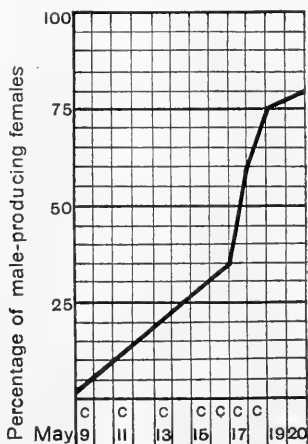


Diagram 2 *Brachionus bakeri*. Experiment 1 of table 2. Showing the production of a high percentage of male-producing females when the food conditions were changed by the additions of considerable quantities of the green flagellate, *Chlamydomonas*. 'C' indicates the addition of the *Chlamydomonas*.

quantities of *Chlamydomonas* were then added to the jars and soon the number of females carrying female eggs decreased and the number of females carrying male eggs increased. In one jar which contained many thousand females it was estimated that only about 10 per cent were carrying female eggs and about 90 per cent were carrying male eggs. The details of these experiments are shown in table 2, a plotting of experiment 1 is shown in diagram 2, and drawings showing the females with the different eggs attached and also of a male are shown in figure 2.

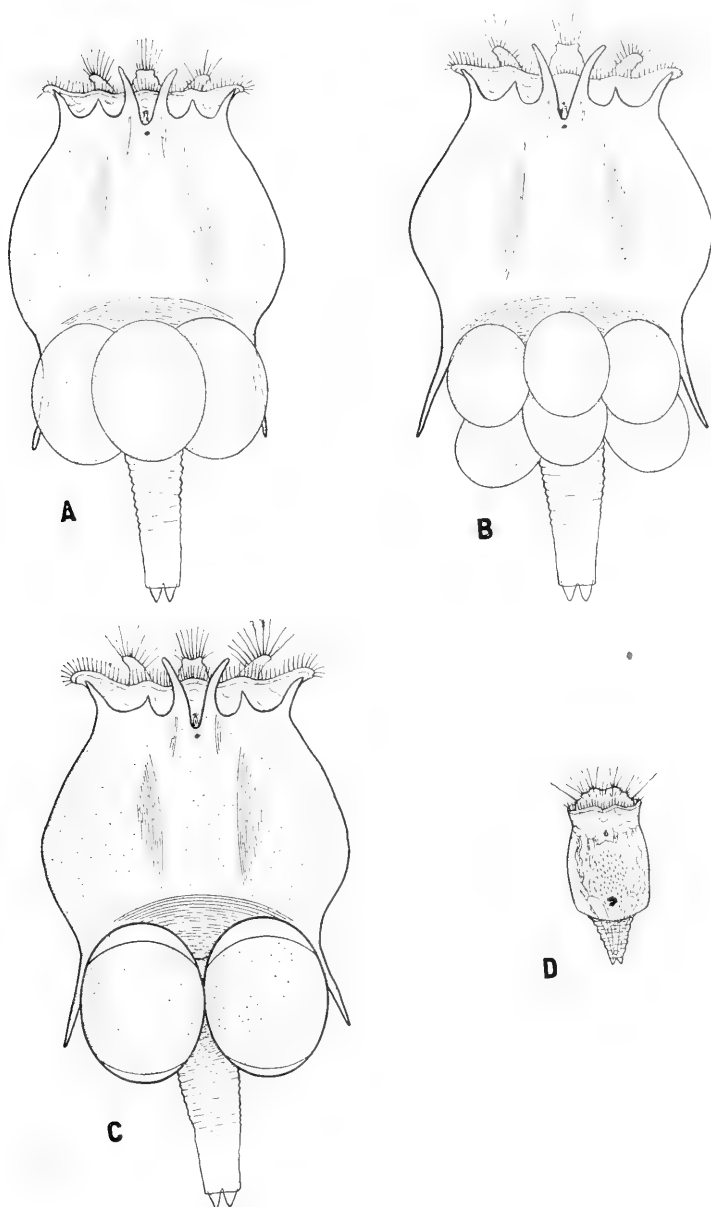


Fig. 2 *Brachionus bakeri* (dorsal views). A, female with attached parthenogenetic female eggs; B, female with attached parthenogenetic male eggs; C, female with attached fertilized eggs; D,  $\sigma^7$  male.

## EUCHLANIS DILATATA

During the spring of 1916 *Euchlanis dilatata* appeared in several of the battery jars of water that had been collected from ponds in the vicinity of Middletown, Connecticut. Small amounts of fresh horse manure and small quantities of *Chlamydomonas* were occasionally added to these jars which were allowed to stand in a large pan of running tap water in a south window. After the jars had become more or less balanced and contained several thousands of the rotifers which were almost exclusively females greater quantities of *Chlamydomonas* were added. Soon males began to appear and continued to increase in numbers until in one experiment they composed 80 per cent of the many thousands of individuals in the jar. When the addition of *Chlamydomonas* was discontinued the males soon disappeared and only females remained. The details of the experiments are shown in table 3, a plotting of experiment 2 is shown in diagram 3, and drawings of a female, male, and the three kinds of eggs are shown in figure 3.

## BRACHIONUS MULLERI

In September of 1916 water containing the marine rotifer, *Brachionus mulleri*, was collected from a salt water lake near Lincoln, Nebraska. Two to three liters of this water were put into each of several battery jars and these were placed near a west window but never received direct sunlight. Into some of these jars a small amount of fresh horse manure was put and into others small quantities of bouillon and stable tea. After these jars had remained undisturbed for several days the rotifers in them were examined and generally all or nearly all of them were found to be carrying female eggs. Then during the following days bouillon or bouillon and stable tea together were frequently added and soon the number of females carrying female eggs began to decrease and females appeared carrying male eggs. In some jars containing many thousands of females the two kinds of females occurred in about equal numbers. In one jar standing in sunlight and containing green microorgan-



TABLE 3

*Mass culture experiments with Euchlanis dilatata showing that when considerable quantities of the green flagellate, Chlamydomonas, were added to the jars of water that many males appeared but when only small quantities of Chlamydomonas or none at all were added very few if any males appeared*

EXPERIMENT	TIME 1916	FOOD CONDITIONS	ESTIMATED NUMBER OF INDIVIDUALS OBSERVED		
			♀	♂	Per cent of ♂
1	April 1	4 liter battery jar of pond water collected. Small amount of horse manure added			
	April 3-May 10	Small quantities of Chlamydomonas added frequently			
	May 10	Considerable quantity of Chlamydomonas added	95	5	5
	May 12		86	20	20
	May 13, 15 and 16	Considerable quantity of Chlamydomonas added			
	May 17		70	30	30
	May 20		125	75	37+
	May 22		297	3	1
	May 23	Considerable quantity of Chlamydomonas added	300	0	0
	May 26	Considerable quantity of Chlamydomonas added	285	15	5
	May 28	Considerable quantity of Chlamydomonas added			
2	May 30		180	120	40
	May 8	4 liter battery jar of pond water collected			
	May 8-May 20	Small quantities of Chlamydomonas added at 48 hour intervals			
	May 20	Considerable quantity of Chlamydomonas added	80	20	20
	May 21	Considerable quantity of Chlamydomonas added			
	May 22		40	160	80
	May 26		160	40	20
	May 30		200	0	0

isms the proportion of females carrying male eggs was in this manner raised to 87 per cent of the total number of females. The details of these experiments are shown in table 4, a plotting of experiment 3 is shown in diagram 4, and drawings of the females with the different eggs attached and also of a male are shown in figure 4.

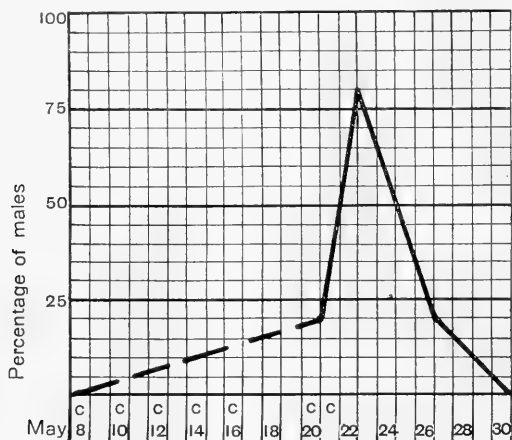


Diagram 3 *Euchlanis dilatata*. Experiment 2 of table 3. Showing the production of a high percentage of males when the food conditions were changed by the additions of quantities of the green flagellate, *Chlamydomonas*. C indicates the addition of large quantities and c indicates the addition of small quantities of *Chlamydomonas*. The broken line indicates an assumed record.

#### THE INFLUENCE OF GREEN FOOD IN PRODUCING MALE-PRODUCING FEMALES IN THE LIGHT AND IN DARKNESS

In 1915 experiments were carried on with the New Jersey *Hydatina senta* in which air was forced through the culture water in which the rotifers and *Chlamydomonas* were living. Some of these cultures were in direct sunlight and some were placed inside of a dark incubator which had an opaque door. All experiments were maintained at a temperature of about 25°C. Other cultures of the rotifers and *Chlamydomonas* were placed inside the incubator but no air was forced through the culture water. Some of the aerated mothers in the sunlight produced 84 per cent of male-producing daughters but in experiments of a former

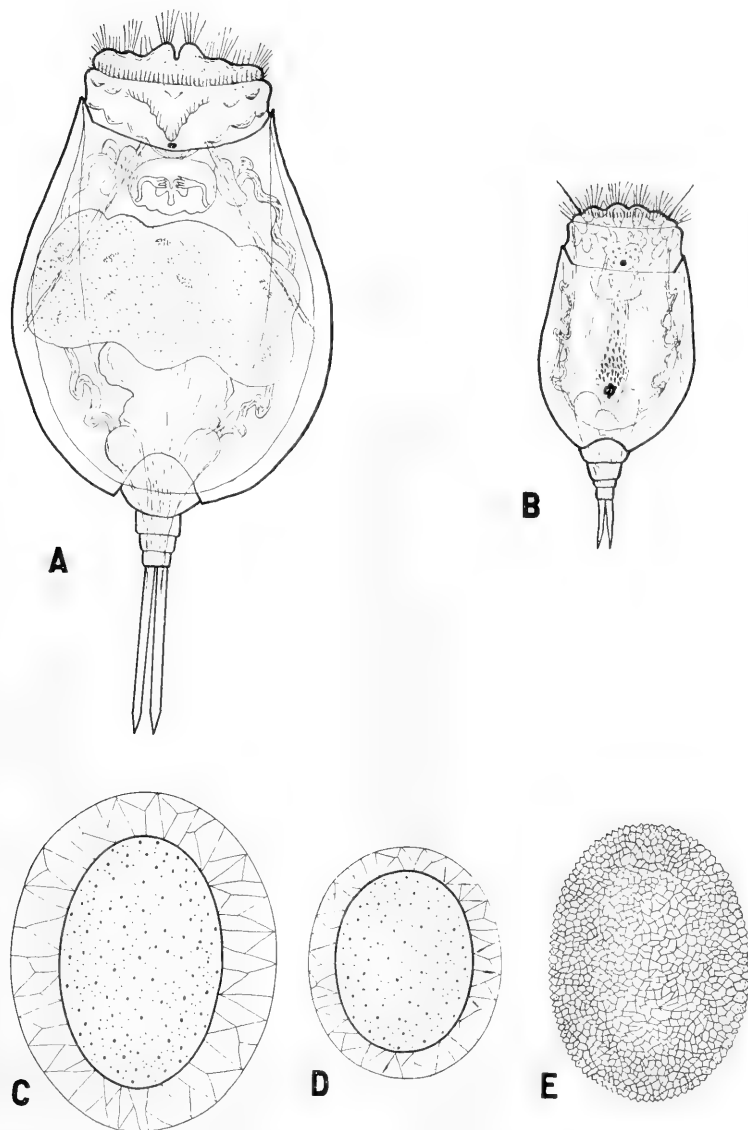


Fig. 3 *Euchlanis dilatata*. A, ventral view of female; B, ventral view of male; C, parthenogenetic female egg; D, parthenogenetic male egg; E, fertilized egg.

TABLE 4

*Experiments with mass cultures of the salt water rotifer, Brachionus mulleri, showing that whenever the colorless or green food supply of micro-organisms is increased or decreased by the addition or withholding of bouillon and stable tea the male-producing females appear in considerable numbers or disappear entirely*

EXPERIMENT	TIME 1916	EXPERIMENTAL SALT WATER POND CULTURES MAINTAINED BY FREQUENTLY ADDING SMALL QUANTITIES OF BOUILLON			CONTROL SALT WATER CULTURES MAINTAINED BY OCCASIONALY ADDING SMALL QUANTITIES OF BOUILLON			
		Food conditions	Estimated number of females observed			Estimated number of females observed		
			♀ ♀	♂ ♀	Per cent of ♂ ♀	♀ ♀	♂ ♀	Per cent of ♂ ♀
1	September 9	2 liters salt collected containing only colorless micro-organisms. Small amount of horse manure added						
	September 23		200	0 0	200	0	0	
	October 14		300	0 0				
	October 16	120 cc. bouillon added						
	October 20	120 cc. bouillon added						
	October 23	12 cc. stable tea added	210	90 30				
	October 24		150	150 50	100	0	0	
	November 1		190	10 5				
2	September 20	3 liters salt water collected containing only colorless micro-organisms. 180 cc. bouillon added	200	0 0	200	0	0	
	September 27		160	210 56+	200	0	0	
	October 5		210	90 30	200	0	0	
	October 7	180 cc. bouillon added						
	October 10	180 cc. bouillon added						
	October 14		500	500 50	300	0	0	
	October 18	180 cc. bouillon added	297	3 1				
	October 20	180 cc. bouillon added						
	October 24		650	350 35	300	0	0	
	November 1		400	2 0.05	100	0	0	
	November 3		1,000	0 0	100	0	0	
3	September 23	3 liters of salt water collected containing only colorless micro-organisms						

TABLE 4—Continued

EXPERIMENT	TIME 1916	EXPERIMENTAL SALT WATER POND CULTURES MAINTAINED BY FREQUENTLY ADDING SMALL QUANTITIES OF BOUILLON	CONTROL SALT WATER CULTURES MAINTAINED BY OCCASIONALLY ADDING SMALL QUANTITIES OF BOUILLON		
			Estimated number of females observed		
			♀ ♀	♂ ♀	Per cent of ♂ ♀
3	September 29	180 cc. bouillon added	300	0 0	300 0 0
	October 2		180	120 40	300 0 0
	October 7		210	90 30	300 0 0
	October 11		270	30 10	300 0 0
	October 16	180 cc. bouillon added	300	0 0	285 15 5
	October 18	180 cc. bouillon added			
	October 20	180 cc. bouillon added	240	60 20	300 0 0
	October 23		150	150 50	300 0 0
	November 1		200	4 2	300 0 0
4	September 16	150 cc. of salt water collected containing green flagellates. 20 cc. bouillon added. 1 cc. stable tea added			
	September 23	1 cc. stable tea added	200	0 0	200 0 0
	October 3	10 cc. bouillon added			
	October 7	10 cc. bouillon added			
	October 9		200	200 50	300 0 0
	October 12		50	350 87+	200 0 0
	October 17	10 cc. bouillon added	99	1 1	200 0 0
	October 20	10 cc. bouillon added			
	October 23		120	180 60	200 0 0
	November 1		300	0 0	300 0 0

paper some unaerated mothers in sunlight produced 87 per cent of male-producing daughters. The mothers aerated in darkness in the midst of *Chlamydomonas* produced only 35 per cent of male-producing daughters. However, some of the unaerated mothers in the midst of green food in the same dark incubator produced as high as 76 per cent of male-producing daughters.

In the sunlight experiments with *Chlamydomonas* shown in the plotted curves of diagrams 1 and 3 and also in many experi-

ments shown in diagrams in a former paper the production of male-producing females practically ceased at the low points of depression in the curves where the food supply was scanty.

In sunlight *Chlamydomonas* being a green organism gives off oxygen in the process of photosynthesis but in darkness no photosynthesis takes place and consequently no oxygen is given off. According to Shull and Ladoff one would expect many male-producing females to be produced in sunlight and *Chlamydo-*

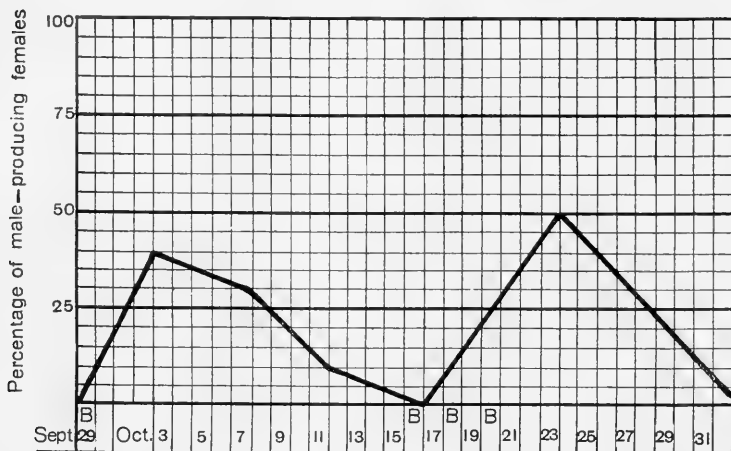


Diagram 4 *Brachionus militaris*. Experiment 3 of table 4. Showing the production of a high percentage of male-producing females when the food of colorless flagellates was increased by the addition of bouillon. B indicates the addition of bouillon.

monas with the accompanying excess of oxygen but no male-producing females would be expected to be produced in darkness and *Chlamydomonas* with no excess of oxygen. In some of these experiments in table 5, 84 per cent of male-producing females were produced in sunlight with oxygen and 76 per cent of male-producing females were produced in darkness without oxygen. This production of such a high percentage of male-producing females in darkness without an excess of oxygen would seem to indicate that oxygen is not the factor that causes this production of male-producing females.

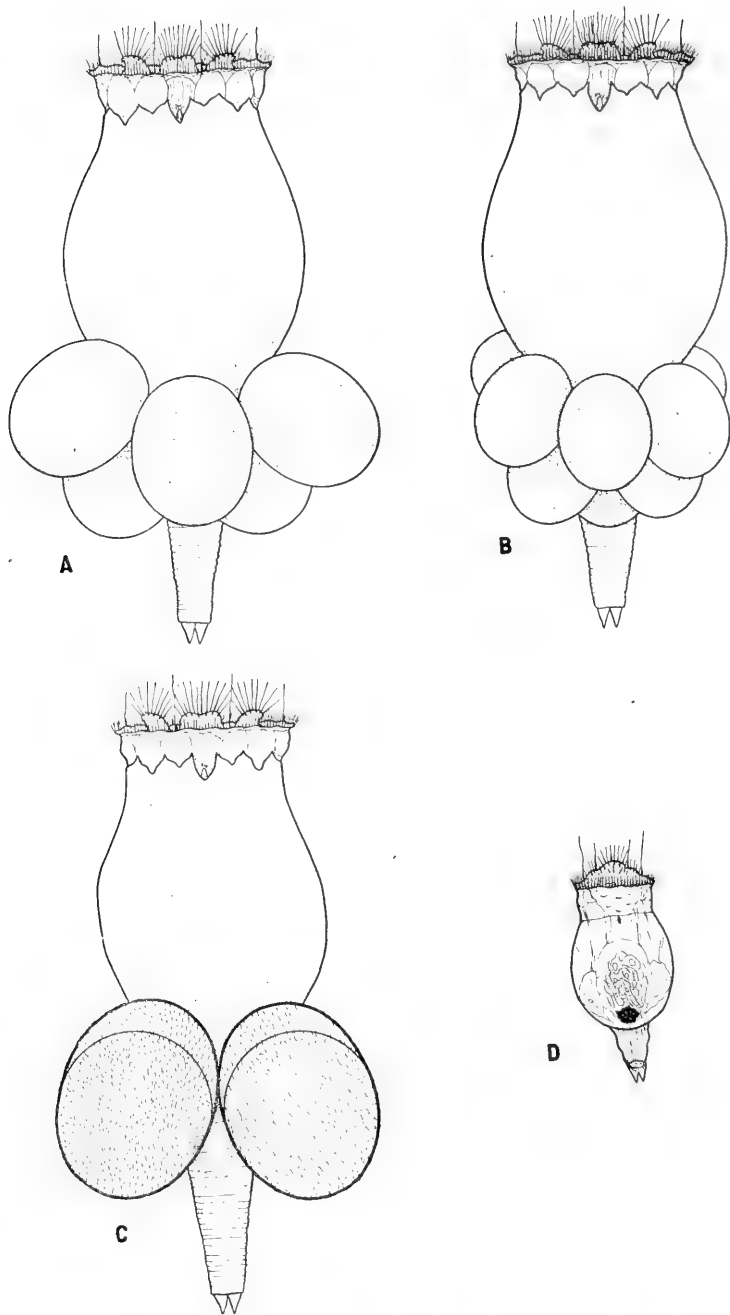


Fig. 4 *Brachionus mulleri* (dorsal views). A, female with attached parthenogenetic female eggs; B, female with attached parthenogenetic male eggs; C, female attached fertilized eggs; D, male.

TABLE 5

*Experiments with the New Jersey Hydatina senta at a temperature of about 25°C. showing that green food will cause mothers to produce male-producing daughters as readily in darkness without an excess of oxygen as in the sunlight with an excess of oxygen present*

	AGE OF GREEN FOOD CULTURES	LIGHT CONDITIONS	DURATION OF AERATION	MOTHERS AERATED	EARLY DAUGHTERS PRODUCED		
					♀ ♀	♂ ♀	Per cent of ♂ ♀
	<i>days</i>		<i>hours</i>				
1	5	Sun	8	5	6	33	84+
2	5	Sun	8	6	7	23	76+
3	7	Sun	12	5	14	20	58
4	14-21	Sun	8	10	60	0	0
5	14	Darkness	12	5	31	9	22+
6	21	Darkness	12	10	52	0	0
7	7	Darkness	12	6	26	14	35
8	7	Darkness	No aeration	5	12	19	61+
9	14	Darkness	No aeration	5	14	5	26+
10	7	Darkness	No aeration	5	13	43	76+
11	30	Darkness	No aeration	5	30	3	9+
12	10	Darkness	No aeration	5	26	12	31+

In the mass culture experiments recorded in this paper and in a former one a high percentage of male-producing females were produced when there was an abundance of growing and reproducing *Chlamydomonas* with its accompanying excess of oxygen in the culture water standing in direct sunlight. As the nutrients of the bouillon or stable tea were being gradually consumed the *Chlamydomonas* gradually ceased reproducing. Some of them went into a quiescent state while others in the adult stage remained more or less motile. When in sunlight during the day they were actively swimming about near the surface of the water but at night they also became quiescent until the sunlight of the next day caused them to become active again. During these days when the *Chlamydomonas* were in these stages just described the rotifers were unable to eat many of them and consequently as they were rather poorly nourished they produced only female-producing females. However, the point to be noted is that during this period there was a formation of gas each day which collected in large bubbles under the scum



on the surface of the water in the jars. This gas caused the glowing end of a burning match to glow more brightly when it was suddenly thrust into a bubble. Presumably this gas was oxygen. However, very few if any male-producing females were produced in these jars in which there was this excess of oxygen.

In experiment 2 of table 6 are shown the results of a balanced culture jar containing green microorganisms and the marine rotifer, *Brachionus mulleri*. At this time about 3 to 4 per cent of the females were carrying male eggs. Compressed oxygen from a cylinder was allowed to pass through this culture for twenty-four hours. This saturation of the culture water with oxygen (perhaps it was already saturated) caused no increase in the numbers of male-producing females thus showing again that oxygen is not a potent factor in the production of male-producing females.

THE INFLUENCE OF OXYGEN UPON COLORLESS ZOOGLEA AND IN CAUSING A CHANGE IN THE RATIO OF THE FEMALE-PRODUCING FEMALES AND THE MALE-PRODUCING FEMALES OF THE NEW JERSEY HYDATINA SENTA

In these experiments an attempt has been made to determine the effect of oxygen upon the protozoa and the bacteria contained in the zooglea. This is considered very essential because if the individuals of the zooglea which are the sole food of the rotifers are increased or decreased the welfare of the rotifers is at once effected. An increase of the individuals in this zooglea might cause better food conditions by increasing or maintaining the number of individuals is to be eaten by the rotifers or it might cause worse conditions for the rotifers by too great crowding or by an accumulation of toxic products from the bacteria.

The zooglea was made by putting about 40 grams of fresh horse manure tied in cheese cloth into a liter of sterilized tap water. This was allowed to stand twenty-four to forty-eight hours and then the old manure was removed and replaced by fresh manure. This process was continued for from one to three weeks. During this time an accumulation of various kinds of bacteria and minute protozoa would form at the surface in a scum. This

TABLE 6

*Experiments with mass cultures of the salt water rotifer, Brachionus mulleri, showing (1) that when oxygen was allowed to pass through the nine day old unbalanced culture water the food supply was increased as was indicated by the greater number of eggs found attached to each female, (2) that when oxygen was allowed to pass through the seventy-nine days old balanced culture water containing many green organisms upon which the rotifers fed there was no increase in the food supply as was indicated by the number of eggs found attached to each female, (3) that a great increase in the food supply caused by adding bouillon produced a larger number of male-producing females than was produced by allowing oxygen to pass through the culture water for twenty-four hours*

EXPERIMENT	TIME 1916	FOOD CONDITIONS	OXYGEN CONDITIONS	ESTIMATED NUMBER OF FEMALES OBSERVED			NUMBER OF EGGS ATTACHED TO THE FEMALE-PRODUCING FEMALES	
				♀ ♀	♂ ♀	Percent of ♂ ♀		
1	November 19	3 liters of salt pond water collected containing miscellaneous protozoa. 200 cc. bouillon added	Bubbles of oxygen allowed to pass through water in jar (10 per minute)	188	12	6	1-2	
	November 20							200 cc. bouillon added
	November 28							
	November 29							
	November 29	Oxygen discontinued	186	14	7	5		
	November 30	Bubbles of oxygen allowed to pass through water in jar (10 per minute)	190	10	5	3-4		
	December 2		198	2	1	1		
	December 3					1-2		
	December 3	Oxygen discontinued						
	December 5	200 cc. bouillon added	198	2	1	1		
	December 7	200 cc. bouillon added	98	15	11	5-6		
	December 9			60	15	20	5-7	
	December 12			100	100	50	5-8	
	December 25			500	0	0	1-2	

TABLE 6—Continued

EXPERIMENT	TIME 1916	FOOD CONDITIONS	OXYGEN CONDITIONS	ESTIMATED NUMBER OF FEMALES OBSERVED			NUMBER OF EGGS ATTACHED TO THE FEMALE-PRODUCING FEMALES
				♀ ♀	♂ ♀	Percent of ♂ ♀	
2	September 11	1 liter of salt pond water collected					
	September 11–November 29	Small quantity of bouillon added occasionally					
	November 29	Many green micro-organisms present	Bubbles of oxygen allowed to pass through water in jar (10 per minute)	288	12	4	1–2
	November 30		Oxygen discontinued	194	6	3	1–2
	December 1			192	8	4	1–2

scum was used as food for the rotifers. Shull and Ladoff used similar feeding methods in their experiments and in order to duplicate their work and to test their results the same kind of food as they used has been used in the present experiments.

In some of the experiments the culture water was first more or less saturated with oxygen by filling a long glass tube with the culture water and connecting this tube to a steel cylinder filled with compressed oxygen and forcing out two-thirds of the culture water and allowing the oxygen to replace that which was forced out. Then the water in the tube was thoroughly shaken in contact with the oxygen. After the shaking was completed the contents of the tube was poured out into a watch glass which was then placed in a Novy anaerobic culture jar. This jar was connected to a vacuum water pump and 40 to 60 per cent of the contained air removed. Immediately after this the jar was disconnected from the water pump and connected to the cylinder of compressed oxygen and the oxygen was allowed to flow into the jar and to replace the air that had been pumped out. After

this the jar was allowed to remain undisturbed for from twenty-four to forty-six hours and then opened and the contents of the watch glasses examined. In other experiments the saturation of the culture water with oxygen in the glass tube was omitted as the results by both methods seemed identical.

The first set of experiments as recorded in table 7 were performed with special reference to the protozoa in the zooglea in order to determine what effect oxygen had on maintaining or increasing the number of individuals in the culture water as compared with other similar culture water subjected to ordinary air conditions. In all the experiments recorded and in many unrecorded ones a decided effect of the oxygen was noted. In all culture waters subjected to oxygen, from eighteen to forty-six hours, many more individual protozoa were counted in a definite microscopic area than in the culture water subjected to air.

The second set of experiments recorded in table 8 were performed in order to determine what effect the oxygen had upon the bacteria in the zooglea on which the protozoa or the rotifers fed in the various waters such as were used by Shull and Ladoff. At the end of twenty-four hours after being subjected to from 40 to 60 per cent of oxygen the culture water in the watch glasses in 16 experiments out of 23 experiments showed a greater number of bacteria per cubic centimeter than did those subjected to air. In one experiment there was no difference and in 6 experiments there were fewer bacteria per cubic centimeter than in the culture water subjected to air. The second important fact determined was that no two watch glass cultures can be made that are identical in food content, as judged by bacterial counts, either at the beginning or at the end of the experiment except when it is done by chance as in lots F and G of experiment 8 in table 8. Before putting the zoogleal scum into the various culture waters about 20 cc. of it was made up with its own culture water in a separate dish and then stirred considerably in order to break up the scum into small fragments. Then 1 cc. of this was added to 4 cc. of the various culture waters. Immediately after this bacterial plates were made and also again at the end of twenty-four hours. The third point to be suggested is that in this

TABLE 7

*Experiments showing that when cultures of various protozoa were subjected to an atmosphere containing 60 per cent oxygen they contained at the end of the experiment a greater number of these protozoa than did other similar cultures which were subjected to ordinary air*

EXPERIMENT	LOT	TIME HOURS	FOOD CONDITIONS	ATMOSPHERIC CONDITIONS	NUMBER OF INDIVIDUALS OF THE UNIDENTIFIED SPECIES OF PROTOZOA COUNTED IN A DEFINITE MICROSCOPIC AREA AT THE END OF EXPERIMENT
1	A	41	Watch glass of 0.07 per cent bouillon to which was added an unidentified species of flagellate	Air	12-16
	B	41	Watch glass of 0.07 per cent bouillon to which was added an unidentified species of flagellate	60 per cent oxygen	40-50
2	A	41	Watch glass of water from general culture jar containing the unidentified flagellates	Air	8-10
	B	41	Watch glass of water from general culture jar containing the unidentified flagellates	60 per cent oxygen	16-25
3	A	46	Watch glass of water from a general culture jar containing unidentified amoebae	Air	2-3
	B	46	Watch glass of water from a general culture jar containing unidentified amoebae	60 per cent oxygen	8-9
4	A	18	Watch glass of 0.1 per cent bouillon to which was added a colorless unidentified species of flagellate but different from the one used in experiment 2	Air	30-40

TABLE 7—Continued

EXPERIMENT	LOT	TIME	FOOD CONDITIONS	ATMOSPHERIC CONDITIONS	NUMBER OF INDIVIDUALS OF THE UNIDENTIFIED SPECIES OF PROTOZOA COUNTED IN A DEFINITE MICROSCOPIC AREA AT THE END OF EXPERIMENT
4 {	B	18	Watch glass of 0.1 per cent bouillon to which was added a colorless unidentified species of flagellate but different from the one used in experiment 2	60 per cent oxygen	70-80
	A	46	Watch glass of 0.1 per cent bouillon to which was added the flagellate, Poly-toma	Air	3-4
5 {	B	46	Watch glass of 0.1 per cent bouillon to which was added the flagellate, Poly-toma	60 per cent oxygen	8-10

zooglea of miscellaneous bacteria the species of predominating bacteria may vary with the age of the zooglea. This is indicated in experiment 5 in which all the rotifers died rather quickly, presumably from some toxic poisoning produced by the bacteria in this older scum. The fourth point determined is that no one can say in advance how well the rotifers in such irregular cultures are going to thrive. As no two watch glass cultures can be made identical in regard to their food contents in species or in number of bacteria there can be expected no uniformity of their effects upon the rotifers in producing male or female-producing daughters. This is amply illustrated by the experiments in table 8 and also in many of the experiments of Shull and Ladoff. The fifth point demonstrated is that male-producing females of the New Jersey strain of *Hydatina senta* are produced equally well under ordinary air conditions as under a 40 to 60 per cent excess of oxygen.

TABLE 8

*Bacterial counts showing (1) that in 16 lots out of 22 lots a 40 to 60 per cent increase in oxygen causes a larger number of bacteria per cubic centimeter than in air, (2) that in 6 lots out of 22 lots a 40 to 60 per cent increase in oxygen causes a smaller number of bacteria per cubic centimeter than in air, (3) that under apparently identical conditions the bacterial count in some cultures is increased and in other cultures it is decreased, (4) that in several dishes apparently inoculated with the same amount of bacterial scum the number of bacteria at the beginning or at the end of the experiment may vary greatly, (5) that male-producing females are produced regardless of the number of bacteria present, (6) that male-producing females may be produced regardless of whether air or oxygen in an abundance is present*

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
1	A	24	5 cc. of 0.1 per cent bouillon plus scum 46 days old	Air		115,000,000				
	B	24	5 cc. of 0.1 per cent bouillon plus scum 46 days old	60 per cent oxy- gen		140,000,000				
2	A	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	Air		13,000,000	5	9	0	0
	B	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	60 per cent oxy- gen		24,000,000	5	9	0	0
3	A	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	Air		5,500,000	1	2	0	0
	B	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	60 per cent oxy- gen		9,000,000	1	3	0	0
	C	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	Air		13,000,000	3	7	0	0

TABLE 8—Continue

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTETS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
3	D	24	4 cc. of 0.1 per cent bouillon plus 1 cc. scum 6 days old	60 per cent oxy- gen	21,000,000	26,000,000	3	9	0	0
	A		4 cc. tap water plus 1 cc. scum and polytoma 9 days old							
	B	24	4 cc. tap water plus 1 cc. scum and polytoma 9 days old	Air		8,000,000	5	3	7	70
4	C	24	4 cc. tap water plus 1 cc. scum and polytoma 9 days old	60 per cent oxy- gen		34,000,000	5	10	0	0
	D	24	Same as in A but plus 5 drops of stable tea	Air		106,000,000	5	10	0	0
	E	24	Same as in A but plus 5 drops of stable tea	60 per cent oxy- gen		27,000,000	5	9	1	10
	F	24	4 cc. of 0.7 per cent bouillon plus same scum as in A	Air		38,000,000	5	9	1	10
	G	24	4 cc. of 0.7 per cent bouillon plus same scum as in A	60 per cent oxy- gen		35,600,000	5	10	0	0
5	A		4 cc. tap water plus a little new scum and polytoma. Plus old thick scum		105,000,000					



TABLE 8—Continued

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS. SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
5	B	24	4 cc. tap water plus a little new scum and polytoma. Plus old thick scum	Air		57,000,000	Died			
	C	24	4 cc. tap water plus a little new scum and polytoma. Plus old thick scum	60 per cent oxy- gen		87,000,000	Died			
	D	24	Same as A but plus 5 drops of stable tea	Air		150,000,000	Died			
	E	24	Same as A but plus 5 drops of stable tea	60 per cent oxy- gen		197,000,000	Died			
	F	24	4 cc. 0.7 per cent bouillon plus scum as in A	Air		245,000,000	Died			
	G	24	4 cc. 0.7 per cent bouillon plus scum as in A	60 per cent oxy- gen		218,000,000	Died			
6	A		4 cc. tap water plus 1 cc. scum and polytoma 7-14 days old		316,000,000					
	B	24	4 cc. tap water plus 1 cc. scum and polytoma 7-14 days old	Air		217,000,000	5	4	6	60
	C	24	4 cc. tap water plus 1 cc. scum and polytoma 7-14 days old	60 per cent oxy- gen		150,000,000	5	8	2	20

TABLE 8—Continued

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS. SCUM FROM STABLE TEA CULTURES	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
6	D	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	Air		212,000,000	5	4	6	60
	E	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	40 per cent oxy- gen		215,000,000	5	8	0	0
	F	24	4 cc. 0.7 per cent bouillon plus scum as in A	Air		204,000,000	5	10	0	0
	G	24	4 cc. 0.7 per cent bouillon plus scum as in A	40 per cent oxy- gen		320,000,000	5	8	2	20
	H	24	4 cc. tap water. Scum as in A plus 3 drops of stable tea	Air		455,000,000	5	19	1	5
	I	24	4 cc. tap water. Scum as in A plus 3 drops of stable tea	40 per cent oxy- gen		500,000,000	5	14	6	30
7	A		4 cc. tap water plus 1 cc. scum with polytoma 9 days old		230,000,000					
	B	24	4 cc. tap water plus 1 cc. scum with polytoma 9 days old	Air	108,000,000	5	10	0	0	
	C	24	4 cc. tap water plus 1 cc. scum with polytoma 9 days old	40 per cent oxy- gen	204,000,000	5	7	1	12+	
	D	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	Air	87,000,000	5	8	0	0	
	E	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	40 per cent oxy- gen	190,000,000	5	9	1	10	

TABLE 8—Continued

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS. SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
7	F	24	4 cc. 0.7 per cent bouillon plus scum as in A	Air		235,000,000	5	10	0	0
	G	24	4 cc. 0.7 per cent bouillon plus scum as in A	40 per cent oxy- gen		260,000,000	5	10	0	0
	H	24	4 cc. tap water plus 3 drops of stable tea	Air		162,000,000	5	17	1	5+
	I	24	4 cc. tap water plus 3 drops of stable tea	40 per cent oxy- gen		193,000,000	5	12	0	0
8	A		4 cc. tap water plus scum with polytoma 10- 14 days old		58,000,000					
	B	24	4 cc. tap water plus scum with polytoma 10- 14 days old	Air		18,400,000	5	10	1	9+
	C	24	4 cc. tap water plus scum with polytoma 10- 14 days old	40 per cent oxy- gen		58,000,000	5	10	0	0
	D	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	Air		40,000,000	5	8	2	20
	E	24	4 cc. N/600 CaCl <sub>2</sub> plus scum as in A	40 per cent oxy- gen		39,000,000	5	10	0	0
	F	24	4 cc. 0.7 per cent bouillon plus scum as in A	Air		70,000,000	5	10	0	0

TABLE 8—Continued

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS. SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
8	G	24	4 cc. 0.7 per cent bouillon plus scum as in A	40 per cent oxy- gen		70,000,000	5	10	0	0
	H	24	4 cc. tap water. Scum as in A plus 3 drops of stable tea	Air		75,000,000	5	9	1	10
	I	24	4 cc. tap water. Scum as in A plus 3 drops of stable tea	40 per cent oxy- gen		37,000,000	5	9	1	10
9	A	24	8 cc. tap water plus 2 cc. of medium scum 3 days old	Air	80,000	35,000	5	20	0	0
	B	24	8 cc. tap water plus 2 cc. of medium scum 3 days old	Air	100,000	29,00	5	19	1	5
	C	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	130,000	23,000	5	19	1	5
	D	24	8 cc. 0.005 per cent creatin plus scum as in A	40 per cent oxy- gen	170,000	56,000	5	16	4	20
10	A	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	24,200,000	29,700,000	5	10	0	0
	B	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	28,000,000	17,200,000	5	10	0	0
	C	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	26,000,000	64,000,000	5	8	2	20

TABLE 8—Continued

EXPERIMENT	LOT	TIME, HOURS	FOOD CONDITIONS. SCUM FROM STABLE TEA CULTURE	ATMOSPHERIC CONDITIONS	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT BEGIN- NING OF EX- PERIMENT	NUMBER OF BAC- TERIA PER CU- BIC CENTIME- TER AT END OF EXPERIMENT	MOTHERS USED IN EXPERIMENT	DAUGHTERS ISOLATED		
								♀ ♀	♂ ♀	Per cent of ♂ ♀
10	D	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	14,000,000	22,500,000	5	8	2	20
	A	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	47,000,000	21,000,000	5	9	1	10
11	B	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	89,000,000	14,000,000	5	10	0	0
	C	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	45,500,000	3,600,000	5	10	0	0
	D	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	90,000,000	2,500,000	5	10	0	0
12	A	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	80,000,000	32,000,000	5	9	1	10
	B	24	8 cc. tap water plus 2 cc. scum 1-2 days old	Air	450,000,000	14,800,000	5	10	0	0
	C	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	22,500,000	73,000,000	5	9	1	10
	D	24	8 cc. 0.005 per cent creatin plus scum as in A	Air	100,000,000	59,600,000	5	10	0	0

TABLE 9

*Bacterial counts in cultures made from tap water and vigorous growing Polytoma cultures, reared in one part stock stable tea and three parts tap water, showing a great decrease in the number of living bacteria during an interval of forty-eight hours under ordinary laboratory air conditions*

EXPER- MENT	LOT	FOOD CONDITIONS	BACTERIA PER CUBIC CENTIMETER AT BE- GINNING OF EXPERI- MENT	BACTERIA PER CUBIC CENTIMETER AT END OF 24 HOURS	BACTERIA PER CUBIC CENTIMETER AT END OF 48 HOURS
1	A	6 cc. tap water plus 3 cc. polytoma cul- ture	65,000,000	Bacterial plates contaminated	150,000
	B	6 cc. tap water plus 3 cc. polytoma cul- ture	58,000,000	Bacterial plates contaminated	140,000
2	A	6 cc. tap water plus 3 cc. polytoma cul- ture	Bacterial plates contaminated	7,000,000	95,000
	B	6 cc. tap water plus 3 cc. polytoma cul- ture	Bacterial plates contaminated	3,670,000	210,000

## GENERAL DISCUSSION

It has been shown in a former paper together with the present one that in nine different species of rotifers the production of male-producing daughters can be controlled by the food conditions. In all of the nine species the production of male-producing daughters can be brought about by feeding the mothers a sufficient quantity of certain green microorganisms in the sunlight.

Shull and Ladoff suggest and contend that the oxygen given off by these green microorganisms during photosynthesis in the sunlight is an important factor in causing the mothers to produce male-producing daughters.

Several years ago it was noted that in 2-4 liter battery jars of newly made stable tea cultures of miscellaneous colorless microorganisms and *Hydatina senta* there was an abundance of male-

producing daughters within a few days after the cultures were made. No green organisms were present nor was there any oxygen being formed in the cultures. Later as the cultures became older and the number of microorganisms in them became fewer the production of male-producing daughters ceased entirely and only female-producing daughters were produced. In some of the present experiments in table 4 *Brachionus mulleri* produced 56 per cent of male-producing females when fed upon an abundance of colorless microorganisms. In some of the control experiments with *Hydatina senta* of Shull and Ladoff in which the mothers were subjected to ordinary air conditions and fed colorless zoogaea each of several mothers produced from 50 to 56 per cent of male-producing daughters. Thus the experiments of Shull and Ladoff together with the author's former ones and some of the present ones agree in that a mother kept under normal air conditions and fed the proper colorless microorganisms can produce over 50 per cent of male-producing daughters.

In table 5 there are also some experiments with *Hydatina senta* which show that when some mothers were put into a dark incubator and fed green food, which could produce no oxygen in the dark, produced 76 per cent of male-producing daughters. Thus showing that with the proper food conditions mothers will produce a high percentage of male-producing daughters in the absence of an excess of oxygen.

The general summaries of many of the experiments of Shull and Ladoff expressed in tabular form would seem to indicate that an excess of oxygen caused male-producing females to be produced. Such also is the conclusion of Shull and Ladoff. However, when one examines each of the several experiments of such a table one sees that nearly all of the male-producing daughters were produced by a few of the mothers while the majority of the mothers produced female-producing daughters although all of the mothers were under the influence of an excess of oxygen. If oxygen has any general influence in causing male-producing daughters to be produced it should show an effect upon a majority of the mothers at least. Table 19 of Shull and Ladoff is here inserted to show that in only three out of eleven experiments

TABLE 19

(From Shull and Ladoff)

*Showing the effect of oxygen and bouillon on one line, as contrasted with a line not subjected to either. Bouillon, even in dilute solutions, has been shown to reduce the number of male-producers (see table 9). In this experiment, oxygen counteracts the effect of the bouillon, and actually increases the proportion of male-producers above that in the control line*

SPRING WATER, WITHOUT OXYGEN			BOUILLON, WITH OXYGEN <sup>1</sup>		
Number of experiment	Number of ♂ ♀	Number of ♀ ♀	Number of experiment	Number of ♂ ♀	Number of ♀ ♀
A	3	23	A	1	24
B	1	11	B	10	4
C	1	23	C	0	8
D	0	14	D	0	3
E	0	16	E	0	23
F	0	26	F	0	12
G	0	13	G	0	9
H	1	22	H	6	12
I	0	3	I	0	11
J	0	22	J	14	20
K	0	6	K	0	2
Total.....	6	179		31	128
Percentage of ♂ ♀	3.2			19.5	

<sup>1</sup> 40 per cent oxygen. Author.

was oxygen apparently effective. Why was not the oxygen effective in the other eight experiments?

The experiments including the bacterial counts in table 8 gives the answer to this question. It is seen by the bacterial counts that it is impossible to make or to maintain two identical watch glass cultures in which the rotifers are living. Under ordinary air conditions two watch glass cultures may be made nearly identical and may remain nearly identical for twenty-four hours, while another set of two watch glass cultures may be practical identical when made but at the end of twenty-four hours one of the two may contain only a few bacteria while the other one may contain many more bacteria than at the beginning of the twenty-four hour period. Furthermore, if two cultures are made as nearly identical as possible at the beginning of the



experiment and one is allowed to stand in ordinary air conditions and the other one is put under an excess of oxygen it seems a matter of chance just what will happen. In a majority of the experiments the cultures under the influence of oxygen contained many more organisms at the end of twenty-four hours than did the cultures under air conditions but in some experiments the cultures under oxygen conditions contained fewer bacteria than those under air conditions. Whether the bacteria furnish the food for the protozoa in the zooglea and the protozoa are eaten by the rotifers or whether the rotifers eat both the bacteria and the protozoa is immaterial. However this may be, the bacteria are the original source of food supply for the rotifers in these cultures of colorless microorganisms.

With the present technique of manipulating the rotifers and their food cultures of zooglea it seems to be a matter of chance what the food conditions will be both at the beginning and at the end of a parallel series of experiments extending through twenty-four hours either under air conditions or under oxygen conditions. This, of course, can be readily explained as being due to the chance development and ascendancy of any of the various species of bacteria in the zooglea.

Table 16 from the paper of Shull and Ladoff is here inserted to show a fine example of this chance development of optimum food conditions.

Under air conditions nine mothers out of a total of twenty-two mothers produced nearly all of the male-producing daughters and under 60 per cent oxygen conditions seven mothers out of a total of twenty mothers produced nearly all of the male-producing daughters. The total summary shows those mothers which were under air conditions produced more male-producing daughters than did those mothers which were under oxygen conditions. Opposite results are shown in the present inserted table 19 of Shull and Ladoff. Why do these experiments disagree? Shull and Ladoff assert that it is because one was a New Jersey race and the other was a Nebraska race of *Hydatina senta* that was used in the respective experiments! It is conceivable that if the experiments of Shull and Ladoff in

TABLE 16  
(From Shull and Ladoff)

Showing number of male-producers ( $\sigma$  ♀) and female producers (♀ ♀) in two lines of *Hydatina*, one reared in water in ordinary air, the other in water saturated with an atmosphere of which 60 per cent was oxygen

AIR				60 PER CENT OXYGEN			
Number of generation	Date of first young	Number of $\sigma$ ♀	Number of ♀ ♀	Number of generation	Date of first young	Number of $\sigma$ ♀	Number of ♀ ♀
	<i>June</i>				<i>June</i>		
1	1	1	20	1	1	4	31
2	3	0	3	2	3	0	9
	4	0	1	3	5	2	22
	6	3	13		5	4	25
3	7	12	10	4	7	3	37
4	9	10	31	5	9	4	17
5	11	0	10	6	11	1	3
6	14	0	5	7	14	5	28
	14	0	1	8	17	16	16
	14	2	28	9	19	3	42
7	15	13	23	10	20	1	8
8	17	23	20		21	11	38
9	19	3	26	11	22	19	31
10	21	15	23	12	25	7	31
11	23	1	46	13	27	11	23
12	25	15	36	14	28	14	25
13	27	18	34	15	30	2	25
14	29	4	45		<i>July</i>		
15	30	6	32	16	2	6	11
	<i>July</i>			17	4	1	30
16	3	24	24	18	6	35	12
17	4	9	39				
18	6	24	29				
Total .....		183	499			149	464
Percentage of $\sigma$ ♀		26.8				24.3	

table 19 had been more extended that they would have had a total summary similar to those in table 16 or vice versa.

The real point of importance not only in these experiments of Shull and Ladoff but also in the present ones with air and oxygen is to determine what influence caused these few mothers in both the air and the oxygen experiments to produce a high percentage of male-producing daughters while the other mothers

under conditions as nearly identical as is possible to make them produced no male-producing daughters. As has been pointed out before the food content, judged by the number of contained bacteria of the different watch glasses is scarcely ever identical at any time during the experiment. Oxygen usually favors an increase in the number of bacteria but if too many bacteria are produced they or their products interfere with the normal metabolism and reproduction of the rotifers. If, on the contrary, a sufficient number of bacteria are not maintained in the culture waters the rotifers are deprived of more or less of food supply. It is quite possible that in these conglomerate mixtures of bacteria in the zooglea all kinds of bacteria are not useful directly or indirectly as food for the rotifers but that only certain species are useful. As there are so many millions of individuals in a watch glass at the beginning of the experiment it is very likely a matter of chance which species will survive and maintain itself or will even increase its own numbers. As a matter of chance some of the watch glass cultures in both the air and the oxygen experiments develop those species of bacteria that cause favorable food conditions for the rotifers to produce male-producing daughters while other cultures in the same series of experiments do not develop those bacteria which cause favorable food conditions for the rotifers and consequently only female-producing daughters are produced.

A fine illustration showing the relative effect of oxygen and food in causing male-producing females to be produced is seen in experiment I of table 6. The rate of metabolism and reproduction was easily ascertained by noting the increase or decrease in the number of eggs found attached to each female-producing female. When there was an abundance of food each female might carry five to eight eggs but when there was only a moderate or scanty amount of food a female-producing female would only carry one to two eggs or if food was too scanty she would carry no eggs at all. When oxygen was allowed to pass through the jar of culture water containing miscellaneous protozoa and the rotifers for twenty-four hours the females soon began to produce more eggs. Before the oxygen was allowed to

pass through the water the females were carrying one to two eggs but within a few hours after the oxygen began to pass through the water each female was seen to be carrying three to five eggs. The oxygen passing through the culture water may have caused a more rapid reproduction of the protozoa or it may have caused all the protozoa in the jar to have become more accessible to the rotifers. It also, of course, produced optimum oxygen conditions for all life processes of the rotifers. Before the oxygen was allowed to pass through the culture water the rotifers remained near the surface and thus were able to eat only such protozoa as were at the surface but as soon as the culture water became saturated with oxygen the rotifers became distributed more or less uniformly throughout the culture water, living as readily at the bottom as at the surface of the culture water. During this period of increased egg production in culture water saturated with oxygen the number of male-producing daughters increased only about 1 per cent or less thus showing the negligible influence of oxygen in causing male-producing daughters to be produced.

When, however, bouillon was repeatedly added to this same culture water a few days after the oxygen experiment had been finished the number of bacteria and of protozoa were so enormously increased that the rotifers being furnished with such an abundant food supply began producing more eggs until some of the female-producing females were found to have as many as eight eggs attached to their bodies. Soon there appeared in this culture male-producing females which rapidly increased in numbers until they formed 50 per cent of the total population of the jar. The contrasting results obtained by oxygen and by food on the same rotifers in the same culture water demonstrates plainly which factor is the influential one.

#### SUMMARY

1. When the green food supply of *Chlamydomonas* was made very abundant the rotifers *Brachionus militaris*, *Brachionus bakeri*, and *Euchlanis dilatata* produced a high percentage of male-producing daughters but when the green food supply was

allowed to become scanty very few if any male-producing daughters were produced.

2. When either the colorless or the green food supply was made very abundant the marine rotifers *Brachionus mulleri*, produced many male-producing daughters but when such a food supply was allowed to become scanty very few if any male-producing daughters were produced.

3. The mothers in a race of New Jersey *Hydatina senta* when fed upon *Chlamydomonas* in the dark without an excess of oxygen present produced male-producing daughters nearly as readily as those mothers which were fed upon the *Chlamydomonas* in the light with an excess of oxygen present.

4. Culture waters subjected to an excess of from 40 to 60 per cent of oxygen in the atmosphere for from eighteen to forty-six hours caused an increase in numbers of the various kinds of protozoa living in such treated culture waters.

5. Culture waters subjected to an excess of from 40 to 60 per cent of oxygen in the atmosphere for twenty-four hours caused an increase in the number of bacteria present in the culture water at the end of the majority of the experiments.

6. Bacterial counts show that a series of culture dishes in an experiment can not be made identical except by chance either at the beginning or at the end of the experiment although all dishes are apparently under identical conditions,

7. With such miscellaneous mixtures of species of bacteria and protozoa in the zoogaea it is largely a matter of chance when they are put into various kinds of more dilute culture waters which bacteria and protozoa will survive or flourish and develop such food conditions as will cause the rotifer mothers to produce either male-producing or female-producing daughters.

8. An excess of oxygen in the culture water does not directly affect a mother and cause her to produce male-producing daughters but by affecting the conditions that influence an increase or a decrease of the microorganisms which constitute the food supply it may indirectly cause her to produce either male-producing or female-producing daughters.

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## THE PEDAL LOCOMOTION OF THE SEA-HARE *APLYSIA CALIFORNICA*

G. H. PARKER

ONE FIGURE

The way in which snails use the foot in locomotion is by no means clear, and the discussion of this subject has led to a number of diverse views. The cilia found covering the foot of some gastropods have been supposed by a few investigators to be the means of locomotion, but the majority of students have maintained that the pedal muscles are the organs concerned with this form of movement. These muscles usually act in rhythmic fashion producing a series of waves that course over the foot, and by this means it is believed that the snail is enabled to move from place to place. The pedal waves of most snails are of small dimensions and the exact way in which they serve in locomotion cannot easily be seen. In the large California sea-hare, *Aplysia californica* Cooper, these waves are of very unusual size and progress at such a rate that they give ample opportunity for the examination of many details which in most snails are quite hidden. I have had the opportunity of studying this animal at the Scripps Biological Institute, La Jolla, California, to the staff of which I am under obligations for many courtesies.

*Aplysia californica* is found in considerable numbers among the rocks at low tide on the beach to the north of the Scripps Laboratory. Full grown individuals average about 25 cm. in length. In such specimens the foot proper is represented by a band running lengthwise the ventral surface and about 23 cm. long by 2 cm. wide. This band, however, is capable of contracting to at least one-half its original length. The locomotor waves, which reach across the whole width of the foot, begin at the anterior

end of that organ and sweep over it posteriorly<sup>1</sup>; they involve not only the pedal muscles proper, but more or less of the musculature of the adjacent body wall. In consequence of the direction and extent of these waves they may be designated as retrograde monotaxis, to use terms introduced by Vlès ('07).

In *Aplysia californica* as a rule there is present on the foot only one wave at a time (fig. 1, *B*), and as this wave dies out at the posterior end, a new one starts at the anterior end (*A*). Occasionally a wave on approaching the posterior limit of the foot decreases its rate of progress and before it has disappeared at the hind end, a second wave makes its appearance at the anterior end thus giving rise to a condition in which two whole waves may be represented on the foot at the same time. This condition, however, is distinctly exceptional, for as a rule not more than one wave can be seen on the foot at any one moment.

In an *Aplysia* whose length of body was about 23 cm., 19 waves passed over the foot in 2 minutes and the animal progressed in that time 124 cm.; or, expressed in averages, a wave appeared every 6.3 seconds and the snail progressed with each wave 6.5 cm. In another *Aplysia* 26 waves passed over the foot in 225 seconds, during which time the snail went forward 135 cm.; or, again expressed in averages, a wave occurred every 8.7 seconds and the animal advanced 5.2 cm. with each wave. It is thus clear that in the ordinary locomotion of *Aplysia californica* wave follows wave about every 6 to 8 seconds and with each wave the snail progresses 5 to 6 cm. In some instances, however, progress was much more marked than these figures indicate; thus an *Aplysia* with a length of body of 23 cm. was seen to advance 8 to 10 cm. for each wave and on one occasion as much as 13 cm. or a little more than half the length of its body. When one compares waves of these dimensions and effectiveness with the small ripple-like movements seen on the foot surfaces of most gastropods, the advantages offered by *Aplysia* for the study of pedal locomotion are evident.

<sup>1</sup> My observations on the direction taken by the pedal waves in *Aplysia californica* agree with those of Jordan ('01, p. 199) on *A. limacina*. Vlès' ('07, p. 277) general statement that in *Aplysia* the pedal waves appear to run from posterior to anterior is very probably inaccurate.



In discussing the locomotion of gastropods two important questions must be considered: first, what part of the foot is at any moment concerned with locomotion, and, secondly, where and how is the foot anchored. On both these questions impor-

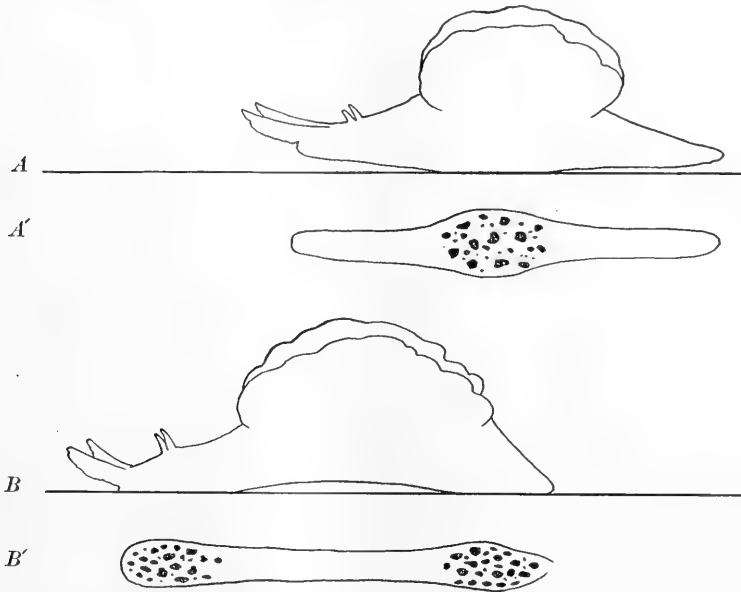


Fig. 1 *A*, side view of an *Aplysia* at the moment the anterior end is raised from the substrate as the initial step in the formation of a wave; the middle of the foot is attached and a wave is disappearing at the posterior end. *A'*, ventral view of the foot, narrow at the two free ends and broad at the attached middle, where fragments of pebbles and fragments of shell are held by suction. *B*, side view at the moment the two ends are attached and the wave has reached the middle. *B'*, ventral view of the foot, showing the narrow free middle and the broad attached ends, to which pebbles and fragments of shells are held by suction.

tant evidence can be had from *Aplysia*, and in dealing with the first of them direct observation is all that is necessary.

A close inspection of the movements of the foot of *Aplysia* is best made on a specimen creeping in a large glass aquarium accessible to observation from several sides. In a specimen so situated each pedal wave can be seen to start at the anterior

end of the foot, as already described, and sweep steadily over this organ to its posterior end, the snail meanwhile making a relatively gigantic forward stride, as Carlson ('05) has described for *Helix dupetithouarsi*. As one wave disappears at the posterior end, another appears anteriorly. At the initiation of locomotion the head of the snail together with the subjacent portion of the foot is lifted well off the substrate (fig. 1, *A*) and projected forward narrowing as it extends till it has been advanced a distance equal to a fourth, a third, or even a half the length of the animal. Then the anterior edge of the foot is brought down on the substrate and attached while the foot posterior to this part forms an arch reaching back to the hind end of the snail (*B*). This end, though still attached to the substrate, is just about to be freed, being in fact the vanishing traces of an area of attachment such as is now established at the front end. The anterior portion of the arch of the foot now crowds forward and attaches itself behind the attached anterior edge and thus the arch itself seems to move backward till the posterior portion of the animal is released from the substrate and is crowded and carried forward a distance equal to that over which the head was advanced. With the disappearance of this wave at the hind end of the snail, a new wave starts at the anterior end and so on in regular succession.

Where on the foot of the creeping gastropod locomotion is actually accomplished, has been a matter of uncertainty. In *Aplysia*, however, there can be not the least doubt that the anterior part of the arch of the foot is the portion that moves forward. The advance of this region and the consequent crowding together of its parts is easily observable, and these changes occur in no other area of the foot. All this is easily seen in *Aplysia*, not only because of the large size of its pedal wave but in consequence of the relatively great height to which the arched portion of its foot is momentarily lifted. In many snails it is very difficult to demonstrate that the moving portion of the foot is lifted at all from the substrate; in fact Biedermann ('05, p. 10), who studied *Halix pomatia* with much care, was erroneously led to believe that this part was actually pressed on

the substrate. But in *Aplysia californica* the arch of the foot is sometimes lifted as much as 1.5 cm. above the surface over which the snail is moving thus enabling an observer when in a favorable position to see completely under the animal. Hence it is perfectly easy to observe that the region of the foot that moves forward is the elevated region and that the most active part of this region is the anterior part.

The second question of importance in the locomotion of gastropods is where and how the foot is attached to the substrate during locomotion. Two methods of attachment have long been known; either the snail holds to the substrate by suction, or a bed of mucus is laid which on the one hand adheres to the substrate and on the other to the animal's foot. In *Aplysia*, as in all other gastropods, the portion of the foot that serves as a holdfast is of course within the limits of the area temporarily applied to the substrate. That part of the *Aplysia* foot temporarily in contact with the ground is formed, as already indicated, by the crowding together of the anterior region of the arch. In consequence of this crowding the part of the foot applied to the substrate is always broader than the portion that is momentarily free from contact. At the moment the middle of the foot is attached and the two ends are free, the foot has the form shown at *A'*, figure 1, broad in the middle and narrow anteriorly and posteriorly. On the other hand, at the moment the middle of the foot is free and the two ends are attached it has the form shown at *B'*, narrow in the middle and broad at the two ends. Thus in the progress of the waves from the head to the tail of *Aplysia* the area including the region of attachment is marked by considerable breadth, whereas that portion of the foot that is free is always narrow.

The means of attachment in the widened portion of the foot can be demonstrated very clearly on an *Aplysia* that is creeping over a shelly or gravelly beach. If such an animal is quickly inverted, the momentarily narrow part of the foot will be found to be quite clean, whereas the broadened part will be seen to be covered with a great number of fragments of shell and gravel, all of which drop off as soon as the region of the foot to which

they are attached narrows. If a fragment of shell or gravel is taken hold of by forceps before it is naturally released, it is found to be held not by its whole surface but by a limited area and with such force as to give the impression that it is held by well localized suction. This view is fully confirmed by the simple experiment of applying one's finger to the widened portion of the foot of an inverted *Aplysia*, whereupon the finger is taken hold of by the foot at several spots and the experimenter has the sensation of strong but local suction at these spots. Since the foot of *Aplysia* is practically free from mucous, it is evident from these observations that its means of attachment is by suction and that this suction is not by the foot as a whole, as in *Crepidula* or *Patella*, but is produced locally.

How local this suction is can be judged from the smallness of the particles of shell or gravel that are found attached to the foot. When an *Aplysia* is picked up from a shelly beach and gently shaken in water, the largest fragments found attached to its foot measure about 1 cm. in diameter. The smallest pieces that are firmly held there have a diameter of approximately 2 mm., thus demonstrating that the suction areas must be of very limited extent. Judging by the distribution of the bits of shell and of gravel, suction may appear anywhere on the full width of the foot and it ordinarily does appear over all that part of the foot that widens in the course of locomotion. In *Aplysia*, then, the widened part of the foot is the part that momentarily serves as a holdfast, and the foot is so organized that its surface can temporarily and locally resolve itself into many sucking organs capable of holding bodies whose diameters are not above 2 mm. Doubtless this local suction is dependent upon the activity of the perpendicular muscle strands in the foot, as surmised by Jordan ('01, p. 197).

In conclusion it may be stated that pedal locomotion in *Aplysia* is due to monotaxic retrograde waves, which lift the foot locally and temporarily from the substrate enabling it thus to move forward with freedom while the rest of the foot for the time being holds the snail in place by many small areas of local suction. The observations on which these conclusions are based

are entirely in accord with the view I have elsewhere expressed as to the mechanics of gastropod locomotion (Parker, '11).

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# THE EFFECT OF TEMPERATURE ON CROSSINGOVER IN DROSOPHILA

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NINE FIGURES

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## I. INTRODUCTION

### *The chromosome theory of linkage*

The phenomenon of linkage between Mendelian characters was discovered in 1906 in the sweet pea. Since that time it has been recognized in a number of plants and in a few animals. By far the most detailed analysis of the phenomenon has been made in a long series of studies on linked mutant characters in the pomace fly, *Drosophila*. It has been shown that in this animal there are four groups of characters, the members of each group showing linkage to each other, but not to the members of any other group. Further than this it has been shown that the relation between the factors in at least three of the four groups, as shown by the percentages of crossingover between them, can be expressed in the form of a linear series. In such a series the factors are represented as being separated by the same number of linear units as the observed percent of crossingover between the characters. Recent work has accumulated a large body of evidence which makes it appear that this is the only system which will fit the observed results. The very obvious similarity between the mechanism which these genetic results require and the chromosomal mechanism makes the identity of these two extremely probable.

### *Variation in linkage intensity*

If the percentage of crossingover observed between any two characters depends on the position of the factors in the chromosome, then the crossover values for those characters should be the same in any experiment done under the same conditions. It is never possible to say that the conditions are even approximately the same unless the parents whose crossingover ratios are to be tested, are sibs, and are mated at the same time, and



kept in the same place while laying. When this is done it is found that the values are uniformly so close as to be within the probable limits of error. When one compares different stocks having the two original characters in different combinations, however, the values often differ by a significant amount. In certain cases this difference can be accounted for, and allowance made. When certain crossover classes have a markedly high viability, it is possible to balance such data by adding more data in which the class in question is a non-crossover class (cf. Balanced Inviability, Morgan and Bridges, '16). In cases where the same cross is made with similar stock, the reason for a statistically significant difference is rather harder to understand. That cases of this sort occur in other forms than *Drosophila* is probable from the variations in linkage suggested by the results of Baur in *Antirrhinum*, Punnett in the sweet pea, and Tanaka in the silk-worm moth (as pointed out by Sturtevant, '14). Two such cases have been analyzed in *Drosophila*. Sturtevant ('17) has recently shown that three definite Mendelian factors occur which influence the amount of crossingover between other factors, while the work of Bridges with second and third broods has made it plain that the amount of crossingover in the second chromosome bears some relation to the age of the female parent.

A significant difference in the amount of crossingover is often shown, however, between cultures which are genetically alike, and which have been laid by a female of the same age. A specific example may make this clear. The summaries given in table 1 show the results of two control series. They were made up in the same way, from the same stock cultures, kept in the same place at room temperature, and counted after the same interval, but one set was recorded about a month later than the other, so that there was probably a slight variation in temperature—between 20°C. and 25°C.—and possibly some difference in the food. The characters used were black body color, purple eyes and curved wings.

The  $X^2$  value shows that the probability that the two results are the same and due to random sampling is about fifteen in ten thousand. Yet when No. 2, table 1, is compared with another

TABLE 1  
b — pr — c

NUMBER	TOTAL	NON-CROSSOVER	1	2	1-2	I	II
						<i>per cent</i>	<i>per cent</i>
1	3,622	2,655	231	685	51	7.8	20.2
2	3,433	2,694	118	588	33	4.4	18.2

$$X^2 = 15.25$$

series composed of sibs made up at exactly the same time, and under environmental conditions which were not sufficiently different to cause a change in crossingover, we get the result shown in table 4. The  $X^2$  value is here 3.1 and the probability of the two values being the same is 38 in 100, which is well within the probable error. It seems probable, therefore, that some slight difference in the environment must have had some effect on the crossingover percentages. It was in order to test whether the cause of such constantly recurring discrepancies was to be found in slight paralleling changes in the environment that the following research was undertaken.

## II. MUTANT STOCKS AND METHODS

Before describing the methods used for testing the effect of variations in the environment on linkage, a word is necessary about the mutant stocks used in the experiments. In all my earlier work the triple recessive stock called black-purple-curved —b pr c—was used. This stock has black body color, purple eyes, and curved wings. The percentages of crossingover given in the published data for these factors are approximately: black-purple, 5.5 per cent, and purple-curved, 19.0 per cent. These percentages are small enough so that double crossingover within each is relatively rare, a fact which makes it certain that any deviation in the amount of crossingover will be detected. In addition, the characters are very easy to determine, so that the possibility of mistakes in counting is very slight. Later star—S'—a dominant character involving a derangement of the ommatidia of the eye; vestigial wings—vg—a recessive character; and speck—sp—a recessive showing a black spot at

the base of the wing; were used in several experiments. The order of these factors and the approximate percentage of crossingover (corrected for double crossingover) is as follows:

In addition to these second chromosome characters, some tests have been made with a sex-linked—first chromosome stock—vermilion-sable-garnet-forked. These results will be described when the data are examined.

The method used for testing the effect on linkage and crossingover of the variations in the environment is very simple. Virgin sister females from the normal wild stock known as 'Falmouth stock,' collected at Falmouth, Mass., were selected and mated (in pairs) to males of the mutant stock to be used in the experiment. The offspring of such pairs will be hetero-

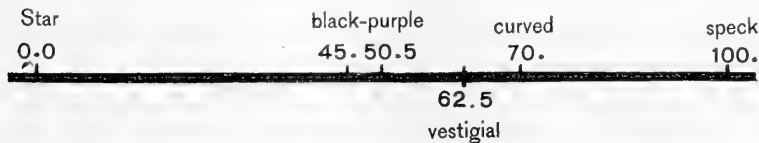


Fig. 1. Diagram of Chromosome II

zygous for the mutant characters in question, and in the developing germ cells of the females hatching from these bottles crossingover takes place. The variations in environmental conditions are therefore applied while these  $F_1$  females are going through their development, and any effect on crossingover will be shown by the percentage of the different classes of the  $F_2$  generation. The results of crossingover are best revealed by back-crossing these  $F_1$  females to males of the original mutant stock. Controls of all experiments are also mated and are generally sibs of the same  $F_1$  pairs tested. The cultures of both control and experimentally treated females are kept under exactly the same conditions and the females allowed to lay for a uniform period of ten days, so that any differential effect of the environment on the viability can not enter into the final result. For the purposes of a more complete analysis this method was varied slightly in later work, but the principle involved remained the same.

## III. NEGATIVE RESULTS

*Moisture*

Before considering the positive results to be recorded, it may be of interest to describe briefly the environmental effects which were tried and found to be ineffective in causing a change in the amount of crossingover. The relative amount of moisture in the food has been observed to have a slight effect on the length of the period between the laying of the eggs and the final hatching of the adults. In a relatively dry culture the adults hatch about two days sooner than those in a very moist culture started at the same time. The flies from the former are also considerably smaller in size than those from the latter. It was conceivable that such conditions might have some effect on the percentage of crossingover. Females from the same stock bottle were mated to black-purple-curved males and placed for four or five days successively in the bottles of Series A, B and C. The bottles of Series A contain food as it is ordinarily used—moist but with no free liquid in the bottom of the bottle—as a control. Those of Series B receive a small amount of food from which as much moisture as possible had been pressed out, and which was sufficiently dry so that no moisture was taken up from it by the paper in which it was wrapped. In Series C, about a quarter of an inch of free liquid was kept constantly on the bottom of the bottle. The results of the backcross testing the percentage of crossingover are given below:

TABLE 2  
b — pr — c

	NUM- BER	TOTAL NUM- BER	NON- CROSS- OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Control.....	A	1,690	1,218	97	345	30	7.5	22.2
Dry.....	B	1,932	1,424	104	374	30	6.9	20.9
Wet.....	C	2,685	1,958	152	534	41	7.2	21.5

The method, admittedly rather crude, shows no significant difference in the amount of crossingover.

*Starvation*

A second test gave no more significant result. Pairs made up as in the previous set of data were successively placed in the bottles of two series, Series A being the control. In the bottles of Series B about one-tenth of the usual amount of food was placed. After several days this food was crowded with larvae and very soon became thoroughly dry. The few female flies which hatched from these cultures had pupated while very small and were under-sized. The back-cross, however, showed little difference in the amount of crossingover.

TABLE 3  
b — pr — c

	NUM- BER	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Control.....	A	729	535	37	150	7	6.0	21.5
Starved.....	B	506	386	23	93	4	5.3	19.1

*Increased fermentation of food*

It was conceivable that the amount of fermentation which the food had undergone might have some effect since this was known to be a rather variable quantity. For the purpose of this experiment the  $P_1$  parents were divided into two groups. Two series were made up with approximately the same amount of the same batch of food in each. Series A was the control, and into the bottles of Series B an even teaspoonful of chemically pure dextrose was placed. The bottle conditions in Series B were markedly better than in Series A, and uniformly larger broods hatched from the former, although approximately the same number of eggs must have been laid in all of the bottles. The crossingover tests of females hatched under these conditions, however, showed differences which have so little significance that the probability that they are the same is 38 in 100. The summaries are as follows:

TABLE 4  
b - pr - c

	NUM- BER	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Control.....	A	3,433	2,694	118	588	33	4.4	18.2
Dextrose.....	B	2,628	2,086	99	422	21	4.5	16.8

$X^2 = 3.1$  - Probability 38 in 100.

### *Iron salts*

The constancy of the strength of linkage was shown further by allowing the  $F_1$  females to develop in food which had been soaked in a solution of the iron salt,  $FeCl_3$ . This was tried in M/12,000 and M/120,000 dilutions with negative results. There is some question, however, as to whether the method permitted action of the salt on the developing larvae and pupae, and therefore the summaries of the counts are not given.

## IV. THE EFFECT OF TEMPERATURE

### *Preliminary experiments*

*Increase in crossingover after both heat and cold.* In sharp contrast to this series of negative results are the striking positive results given by testing the effect of extremes of temperature. These tests were carried on at first by the same methods used in the experiments described above. A set of  $P_1$  parents—black-purple-curved being the recessive mutant stock used—were placed for four or five days in the bottles labeled A, B and C respectively. Series A was kept at room temperature, which varied between 22°C. and 24°C. Series B was kept in a Cyphers electric egg incubator—'Electrobator No. 2'—at 31°C., which is close to the maximum temperature giving fertile offspring, and Series C was kept in a carefully regulated refrigerator at about 13°C. Great care was taken to prevent undue variation in temperature, and in all of the experiments a temperature table was kept and the temperature for each series recorded morning and evening. As was to be expected, the control table showed

the greatest variations in temperature, though it was always kept on top of the incubator in a south room which was kept heated as evenly as possible. For this series the temperature ran ordinarily from 22–24°C., but on one or two occasions reached 20 and 26°C. As will be seen from the results recorded, a variation even between these limits does not effect more than a negligible change in the linkage ratios, and therefore it may be disregarded. In the other two series variation was extremely slight, in the incubator not more than 0.5°C., and in the ice-chest 1°C. in either direction. It was found that the  $P_1$  females did not lay well if placed immediately into the temperature at which their eggs were to be hatched. In both the cold and the heat series, therefore, the bottles were allowed to stand at room temperature for twenty-four hours after the  $P_1$  pairs were introduced before being transferred to the temperature to be tested. This generally insured sufficient eggs to give the required number of  $F_1$  females for the back-cross test, and had no effect on the test itself. It was also found later that it made no difference in what order the test series were made up. The cold test could be taken first, the control second, and the heat last, or in any other combination. The temperature at which the  $P_1$  females laid the eggs affected the number of eggs, but only the temperatures at which those eggs developed and hatched showed an effect on the percentage of crossingover among their offspring. At the control temperature flies begin to hatch in about twelve days, at 12°C. in from three to four weeks, and in the incubator at 31°C. in about ten days. The first test of the effect of temperature gave the following results:

TABLE 5  
b — pr — c

	NUM- BER	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Hatched at 22°.....	A	3,622	2,655	231	685	51	7.8	20.2
Hatched at 31°.....	B	3,547	2,265	333	785	164	14.0	26.7
Hatched at 13°.....	C	2,972	1,854	310	716	92	13.5	27.2

$$A \times B \quad X^2 = 113.5$$

$$A \times C \quad X^2 = 113.5$$

The difference in the percentage of crossingover is so striking as to leave no doubt that it is caused by the change in temperature at which the  $F_1$  females were hatched. These females are offspring of the same females, and as larvae eat practically the same food, but develop at different temperatures and therefore the interval before hatching is shorter or longer as the case may be. The  $X^2$  values for the control with either heat or cold are so great that there is no possibility at all that the differences can be due to random sampling, and it is very obvious that these are greatly in excess of the  $X^2$  values found in an earlier paragraph for the greatest observed difference between controls. The increase in the percentage of crossingover for both B and C is roughly 100 per cent for the first class, or shorter region of the chromosome and 33 per cent for the longer or second region. The fact that longer regions, which permit of greater double crossingover tending to obscure the actual results, show smaller percentages of observed increase, is brought out very clearly in later experiments. The most striking thing about the result as a whole is that both a temperature considerably above and one considerably below the normal give approximately the same result—an increase in the amount of crossingover, or conversely a reduction in the strength of linkage.

The increase caused by heat was confirmed with the different stock containing the mutant characters—black-purple-vestigial. In this case the temperature used for the test was 31.5°C., slightly higher than in the previous experiment. The results as shown by the backcross were as follows:

TABLE 6  
b — pr — vg

	NUMBER	TOTAL	NON-CROSS-OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Control.....	22°C	2,139	1,632	184	293	30	10.0	15.1
Heat.....	31.5°C.	1,099	750	127	183	39	15.1	20.2

The control value for the black-purple region is here much larger than usual, perhaps due to the presence of some modifying



factors, while that for the purple-vestigial region is about that usually found. The rise of slightly more than 5 units in each case is about what we should expect on the basis of the data in the previous experiment.

*Heat effect apparent for short regions only.* The third experiment is instructive. It was made with the characters star—S'—black and curved. Since star is a dominant and the homozygous flies do not survive, it was necessary to make the test by using P<sub>1</sub> star females crossed to P<sub>1</sub> black-curved males. The star F<sub>1</sub> females were then selected and backcrossed to the double recessive black-curved males, and the crossing over percentages among their offspring calculated. From the data of other workers in this laboratory who have tested the star-black region with several intermediate factors, it has been shown that the two are separated by approximately 45 units. As the control value shows this percentage is not realized when no intermediate points are used in the experiment because of the obscuring of the result through unobserved double crossingover. For the same reason the black-curved percentage is not as large as the sum of the black-purple and purple-curved percentages in the first experiment would lead one to expect. The temperature used in this experiment was again 31.5°C. and the test gave the following results:

TABLE 7

S'  $\frac{\quad}{b \quad c}$

	NUMBER	TOTAL	NON-CROSS-OVER	I	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
Control.....	22°C.	6,009	2,827	1,915	872	395	38.4	21.1
Heat.....	31.5°C	3,769	1,528	990	814	437	37.8	33.2
Second broods of heat bottles.....		804	332	257	121	94	43.6	26.8

The second brood data are of interest but will be discussed in another section in connection with other similar data. The first brood data show very clearly that while the black-curved

region of the chromosome shows an unquestionable increase of more than 50 per cent, no increase at all is registered in the test between star and black. This can mean only that with such long distances any increase in the actual amount of single crossingover is compensated by a similar increase in the amount of double crossingover, and thus no increase at all appears in the percentage registered by the count. This fact emphasizes the importance of working with 'short chromosome regions,' that is, with factors between which there is only a low percentage of crossingover, when testing for environmental effects on linkage.

The summaries of bottle counts which have been given so far do not give any idea of the amount of variation between individual bottles in any series, nor of the amount of overlapping between the series. This can be gained in all cases by a comparison of the detailed summaries in the appendix. In view of the large number of flies involved in table 7, it has seemed of value to illustrate the percentage frequencies of the bottle counts in the form of a curve. The results of this assembling may not be as accurate as the weighed mean given in the summary because a large brood has no more weight than a small one, but they tell more about the data given. In each case the standard deviation is lower for the smaller chromosomal region, and the fact bears out the statement already made that the shorter region in every case is the more accurate. The increase in standard deviation of the heat series over the controls is not statistically significant. The fact that the means differ very little from those given in table 7 shows that by either method the increase due to heat is shown with equal clearness.

#### *Complete analysis of temperature effect*

After establishing the fact that extremes of heat and cold acting on the females up to the time of hatching caused a decided increase in the percentage of crossingover in the first ten day broods, it became important to secure material for a table which would show the steps by which this rise took place. To do this series of tests were made, using ten different temperatures ranging from the minimum to the maximum at which

fertile females would hatch. Because of the large amount of labor involved, it was obviously impossible to run these all at the same time, or to secure offspring from the same  $P_1$  parents. Equal care was used in all the tests, however, and careful controls were run with each set. With one exception—No. 6 in the table—the mutant stock used was black-purple-curved, and

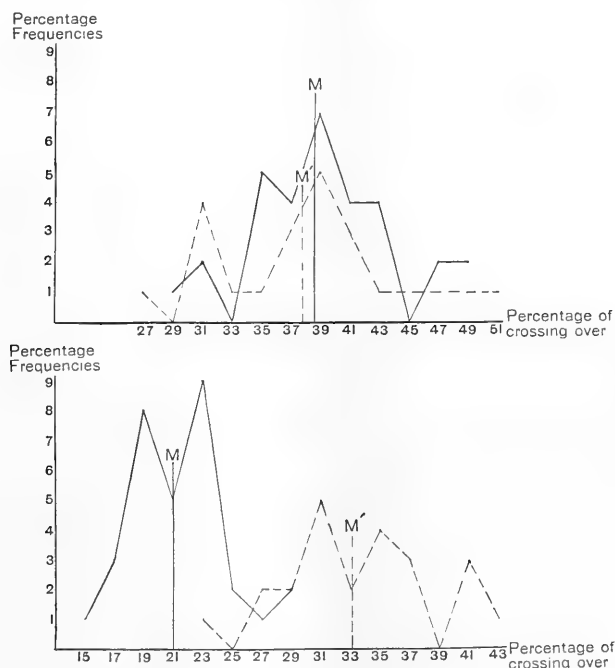


Fig. 2. Percentages of crossingover, for both regions of the bottle counts summarized in table 7—plotted to show variation (cf. Appendix).—— = controls; - - - = ♀ parents hatched at 31.5°.

STAR-TO-BLACK REGION (ABOVE)		BLACK-TO-CURVED REGION (BELOW)	
Control	Heat treated	Control	Heat treated
n = 31.0	n = 23.0	n = 31.0	n = 23.0
M = 38.7	M' = 37.9	M = 21.0	M' = 33.1
±0.59	±0.86	±0.38	±0.69
σ = 4.83	σ' = 6.14	σ = 3.18	σ' = 4.88
±0.414	±0.610	±0.272	±0.485

the  $F_1$  females were allowed to lay for ten days. No. 6 was run in conjunction with another experiment. The characters used were star-black-curved. The data given in No. 2 represents the summaries of the series of counts from flies raised in tubes, the parents being shifted every two days instead of remaining in one bottle for ten. For most of the temperatures tested, a carefully regulated refrigerator and a large and small electric incubator sufficed. The test summarized in No. 4, involving a temperature of  $17.5^{\circ}\text{C}$ . was made by placing the bottles in Dr. A. M. Banta's artificial cave at the Carnegie Institution, Cold Spring Harbor, Long Island. Here the temperature remains nearly constant for long periods, and was observed to vary less than  $1^{\circ}\text{C}$ . during the period of the test. I am indebted to Dr. Banta for the opportunity to use the cold-room during the summer of 1916. No. 5 represents the average of all the controls with the dextrose total added, and is introduced to make the table complete. The temperature was subject to a variation of about  $2^{\circ}\text{C}$ . in either direction. The summaries for successive temperatures are shown in table 8.

The results given in the column at the left are brought out with great clearness when plotted in the form of a curve. For reasons already discussed, the percentages of the shortest region—black-purple—are probably more accurate, and therefore these alone are used for the curve. The actual percentage given in No. 6 cannot be used, for obvious reasons. Because of the fact that the smaller region shows no significant increase over the control, we are justified in continuing the temperature curve for  $22^{\circ}\text{C}$ . through the  $27^{\circ}\text{C}$ . point.

In constructing the curve, the value of the corresponding control must be taken into consideration. It has already been noted that sibs from the same  $P_1$  parents show very little variation in percentage of crossingover, while cultures made up at different times may vary as much as three units for a cross-over percentage of this size. The curve will probably be more accurate, therefore, if the temperature value is increased or decreased in the same proportion that its corresponding control shows an increase or decrease over the mean value for the con-

TABLE 8  
b - pr - c<sup>1</sup>

trol given in No. 5. These weighted values for the black-purple region are given in the center column in table 8.

Alone and unrelated to any other set of data, the differences between No. 4 and No. 5, No 5 and No. 7, and No. 8 and No. 9, would not have great significance. When the table is taken as a whole, with the values properly weighted by their corresponding controls, it becomes clear that the curve presents a consistent picture of the amount of crossingover in the second chromosome between the minimum and maximum points at which fertile offspring can be secured. At 5°C. negative results were shown,

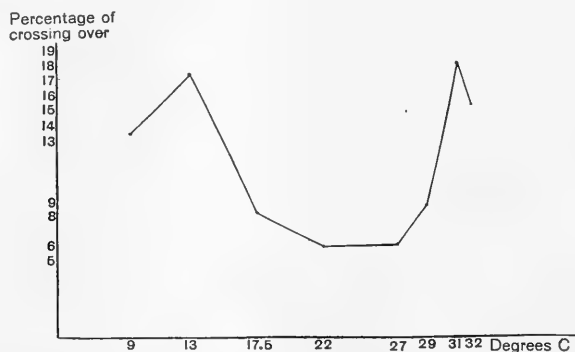


Fig. 3. Weighted values for black-to-purple region in table 8 plotted to show the effect of temperature on crossingover.

for though a few pupae appeared in the bottles, no flies hatched. Similar negative results were given at the high point 35°C., except that in this case several females hatched but on being tested were found to be entirely sterile for the first ten day period. This can mean only that the eggs which went through the growth period while the female parent was pupating were injured in some way by the high temperature and did not develop.

#### *Discussion of temperature curve*

The establishing of such a curve is of some practical value for the reason that it shows the approximate limits in degrees C. between which bottles must be kept to be comparable. When

testing factors in the second chromosome, bottles kept between 19°C. and 27°C. will show a negligible deviation in percentage of crossingover due to temperature, and may be safely compared, other things being equal. This is reassuring to most workers with *Drosophila* for ordinary room temperature seldom varies beyond these limits.

The curve is of very great theoretical interest in another connection. A glance at its form is sufficient to convince anyone that we are not dealing here with an ordinary chemical reaction following van't Hoff's law, such as is commonly found in connection with many physiological processes. The amount of crossingover is not approximately tripled by a rise in temperature of 10°C., as we should expect if the process were comparable to those tested by Snyder, 1908, and other workers in this field. The observed increase in crossingover reaches a maximum at about 13°C. and a second higher maximum at 31°C. Between these two a minimum is reached which continues with little alteration for 8 or 9°. Such a curve would seem to indicate some sort of a change in the physical state, such as might be met with in a study of certain colloidal phenomena.

There is an extremely interesting parallelism between the curve given above for the alteration of the percentage of crossingover and that which physiologists have worked out for the amount of contraction of various types of muscle when given the same stimulus at different temperatures. This fact has been called to my attention by Mr. R. H. Bowen of this laboratory. In his Textbook of Physiology, Howell gives such a curve for the height of contraction of frog's muscle in which the amount of contraction increases gradually from zero degrees Centigrade to 9°C. where it reaches a first maximum. The amount of contraction then decreases, reaching a low point at between 16°C. and 20°C. It then rises rapidly, reaching a higher maximum than the first at about 28°C., after which it continues to decrease until *rigor mortis* sets in at 38°C. Such a striking correspondence, even in the exact temperatures, would seem to be more than mere coincidence.

The facts with regard to stimulation to parthenogenetic development by high and low temperatures should be recalled in this connection. Greeley in 1902 found that a relatively long exposure to low temperatures—0°C. to 6°C.—had a marked activating effect on the eggs of *Asterias*. R. S. Lillie, in 1908, showed that a similar effect resulted from a momentary exposure to high temperatures—30°C. to 35°C. These facts have been recently confirmed by Lillie ('17) in connection with his butyric acid treatment. Between 8°C. and 28°C. little stimulating effect is noted. Lillie states his conclusions with regard to these facts as follows:

In this case of high temperature (above 30°C.) the activating influence is probably to be referred to structural changes in the protoplasmic system, as the temperature-coefficients indicate, and the same appears to be true of cold. Changes in the physical condition of the structural colloids, e.g., gelation, dehydration, altered aggregation-states, may alter locally the permeability or other properties of the protoplasmic system (e.g., of membranes or other barriers to diffusion) and thus render possible interactions which are not possible at ordinary temperatures (Biol. Bull., March, '17).

Whether this work has any bearing on the problem in hand, is an open question. In any case the long series of studies on *Drosophila* from this laboratory have shown beyond a doubt that the chromosomal mechanism is responsible for the crossingover phenomenon. One does no more than restate the facts, therefore, in concluding that an environmental influence, temperature, which probably causes some alteration in the physical basis of the egg has a definite measurable effect on the nuclear phenomena in the developing egg. Just what this effect is and what clue it gives to the nature of the crossingover process, future research may show.

*Failure of heat to cause crossingover increase in presence of 'little crossover' modifier*

Before passing on to an analysis of the extent of the temperature effect, it will be of interest to record one case in which a temperature of 13.5°C. was absolutely ineffective in causing an in-



crease in the percentage of crossingover in the second chromosome. Sturtevant has discovered a definite Mendelian factor which entirely prevents crossingover in the second chromosome between the factors star and black, and considerably reduces it between black and purple. Between purple and curved its presence results in a rather increased percentage over the normal control. This 'little crossover' factor is known as C II 1. It was possible that increased temperature might cause some crossingover in the star-black region. The test was made with characters which involved the whole length of the chromosome so far worked out. In addition to C II 1, star, black, curved and speck were involved in the experiment. A glance at the chromosome map (fig. 1) will show the relative positions of these factors constructed on the basis of numerous experiments with the ordinary stock. The P<sub>1</sub> parents in the test were star-black-males mated to females having C II 1, curved and speck, kindly supplied by Dr. Sturtevant. The star F<sub>1</sub> females were back-crossed to males of the multiple stock black-curved-speck. The results were as follows:

TABLE 9  
S' b  
CII c sp

FEMALES HATCHED AT	TOTAL	S'-b+ *c-sp	S'-b- c-sp + N	S'-b- sp+c	S'-b- *c+sp	S'-c	c-sp
						per cent	per cent
Control, 22°C.....	2,057	917	413	594	133	26.6	35.3
Second broods after 10 days at 22°	1,170	576	216	337	41	21.9	32.3
Heat, 31.5°C.....	1,353	671	249	350	83	24.5	32.0
Second broods after 10 days at 22°	211	108	41	54	8	22.7	29.0

\*No crossovers between S<sup>1</sup> and b in any case.

The summary shows conclusively that far from causing any crossingover in the star-back region the heat treatment apparently accentuates the effect of the 'little crossover' factor. The diminution in the percentage of black to curved in the heat series is not great enough to be very significant but at least it makes it certain that heat does not cause the expected increase, and is not able to overcome the effect of C II 1. The column

giving the percentages of crossingover for the second region—curved to speck—simply emphasizes the conclusion drawn from the data on the star to black region in table 7, namely, that for factors between which there is a high percentage of crossingover, any actual increase in single crossingover is compensated by a similar increase in double crossingover, with the result that no increase at all can be read from the table. The results shown by the second brood counts will be discussed below.

#### V. THE EXTENT OF THE TEMPERATURE EFFECT

##### *Increase in percentage of crossingover not inherited*

If, then, the fact has been established that heat and cold applied to female flies while they are going through their development from egg to adult cause an increase in the percentage of crossingover among the first ten day broods of their offspring, then we can carry the analysis further. It is, of course, a common place of work with *Drosophila* that no crossingover takes place in the germ cells of males. Several tests established the fact that this condition is not altered when the males are subjected to the high temperatures. Males so treated show exactly the same genetic differences as do males which have developed at room temperature. The next question which suggests itself is: What is the extent of the temperature effect as produced on the female germ cells? Such a question must be resolved into two parts: 1) Does the temperature produce any effect on the offspring themselves? 2) Does the temperature affect the entire output of eggs of any one female or simply the brood of the first ten days?

Since the offspring forming the first ten day brood are normal in appearance, the first of the questions proposed means simply, Is the increased percentage of crossingover induced by heat or cold inherited? Counts from a very small number of cultures showed that this question should be answered in the negative. Female offspring of females which had been treated with high temperature behaved genetically exactly as do females hatched from control cultures. Counts of two cultures will make this

clear. The females which laid the first ten day brood recorded were the offspring of a female which had hatched at 31.5°C., No. 430. The first, No. 468, itself hatched from a tube kept at 31.5°C. The second, No. 474, hatched from another tube kept at 22°C. Both were heterozygous for black-purple-curved and were back-crossed to a black-purple-curved male from stock. The results follow:

TABLE 10  
b — pr — c  
P<sub>1</sub> parents hatched at 31.5°

HATCHED AT	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
						<i>per cent</i>	<i>per cent</i>
31.5°—No. 468.....	262	174	16	65	7	8.8	27.5
22° —No. 474.....	190	157	5	27	1	3.1	14.7

The percentages show the characteristic increase in crossing-over among the offspring of the heat-hatched females, while that hatched at 22°C. gives a percentage even lower than normal. The effect of the temperature is shown, therefore, not to be inherited.<sup>1</sup>

#### *Second brood data*

The analysis of the extent of the temperature effect now leads to the second question proposed above, namely, is the entire output of eggs of a female hatched at a high temperature affected or only those laid during the first ten day period? The answer to this question has resulted in a corollary of considerable theoretical importance, and therefore the results will be given in some detail. The method for testing this point is very simple. After the F<sub>1</sub> females backcrossed to males of the mutant stock have been allowed to lay in one bottle for ten days,

<sup>1</sup> The fact that both the control and the heat values given in table 10 are significantly lower than those given in table 8, suggests that possibly high temperatures may result in a lower rate of crossingover among the offspring, though that low rate can be stimulated proportionally by exposure of the parents to heat. The effect of heat continued for many generations should be further investigated.

they are removed and placed in a second bottle for a second period of ten days. A third brood may even be taken in many cases. The results of each of these successive broods are tabulated and compared with the results of the first broods.

As mentioned early in this paper, the results of Bridges have shown that a decrease in the amount of crossingover in the second broods of second chromosome experiments occurs. The data here presented confirm these findings. In table 9 the percentage of crossingover in the first region—which is the only one of great significance—fell off about 5 per cent in the second brood of the controls. The flies which had been subjected to increased temperature need not be considered in this case, since no effect was noted when C II 1 was present. In table 7, however, second brood totals are given for flies which showed a decided increase in crossingover in the first broods due to heat. The percentage for the shorter region—per cent II in the table—shows a falling off of 7 per cent in the second broods. Though this is more than the usual deviation for second broods, this decrease does not bring the percentage down to that of the first brood controls, therefore further evidence on the point is necessary. First and second broods of both control and heat-treated flies from the same  $P_1$  pairs were taken. In addition for both sets of data second broods were taken from flies which were kept at the high temperature rather than at 22°C. while laying the first broods. The results are as follows:

In order to make the significance of this table plain each item will be discussed individually. For Nos. 1 to 4, the stock star-black-curved was used. In these cases, then, only the shorter region—per cent II in the table—is significant. No. 1 was a normal control, the females hatched at 22°, mated and laid their first brood at the same temperature. No. 2, the second brood after this treatment, showed the usual decrease in the amount of crossingover already pointed out by Bridges. No. 3 differed from No. 1 only in that the female parents were hatched at 31.5. These females were mated and laid the first brood at 22°. The values of 29.8 per cent for the black to curved region is more than nine units higher than the control, due to the effect

TABLE 11

S'

b	c
---	---

NUM- BER	DESCRIPTION	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
							<i>per cent</i>	<i>per cent</i>
1	Control bred at 22°.....	1,195	579	371	184	61	36.1	20.5
2	Second brood after 10 days at 22°.....	1,331	761	371	161	38	30.5	14.9
3	Heat 31.5°, bred at 22°....	724	343	165	163	53	30.1	29.8
4	Second brood after 10 days at 22°.....	665	344	202	85	34	35.4	17.8
bl pr cv								
5	Control 22°, bred at 22°...	2,219	1,678	108	409	24	5.9	19.5
6	Second brood after 10 days at 22°.....	966	777	16	171	2	1.8	17.9
7	Third brood after 20 days at 22°.....	726	571	24	127	4	3.8	18.0
8	Second brood after 10 days at 31.5°.....	316	169	44	81	22	20.8	32.6
9	Heat 31.5°, bred at 31.5°...	345	195	42	95	13	15.8	31.3
10	Second brood after 10 days at 31.5°.....	118	79	17	20	2	16.1	18.6

of the heat. The second brood from these parents, No. 4, after ten days at 22°, gave a value very close to the control second broods (No. 2) and twelve units less than the first brood value (No. 3). Such a decided falling off can mean only one thing, namely, that the second brood values for females which had been treated with high temperatures up to the time of hatching, closely approximate the corresponding values for flies not so treated, and that the heat effect is no longer observable after the first ten day period. For Nos. 5 to 10, a different stock was used, black-purple-curved, and both crossingover percentages are significant, though the shorter one—black to purple, per cent I—is the more reliable. No. 6 shows the usual second brood decrease below No. 5, while No. 7 confirms the observation of Bridges that third broods show a tendency to return to the value for the first brood. No. 8, however, gives the values for second broods when the females were kept at 31.5° during the

first ten-day period, after mating. The value is nearly fifteen units higher than the first brood control No. 5, and more than eighteen units higher than the corresponding second brood taken after ten days at 22°, No. 6. Such a striking difference proves conclusively that the percentage of crossingover can be increased in the second brood by simply keeping the parents at the high temperature while they are laying the first, even though that first brood shows no increase whatsoever. This point is more firmly established by No. 9 and No. 10. No. 9 is the first brood taken from females hatched at 31.5° but mated and kept for the first ten days at 31.5°. The usual increase over No. 5 was shown. The second brood, No. 10, was laid at 22°, yet it remains at the high point, instead of dropping as did No. 4, when the first brood of a similar mating was taken at 22°. Taken as a whole, the data in table 11 clearly establish the fact that high temperature is effective in increasing the percentage of crossingover only for the brood taken immediately after the exposure for ten days, and that this exposure may occur either while the female is going through its metamorphosis or after it has reached the adult stage and has been mated.

#### *Fractionations of brood counts*

The value for the black-purple region given in No. 9, table 11, is 15.8, while the value for the same region at approximately the same temperature given in table 8 is 14. The only difference is that the table 9 value was given in a culture kept at 31.5° rather than at 22°. This increase suggests the possibility that some effect of the temperature at which the brood is laid may be registered even within the ten day period of the brood in question. It may mean that the early part of a count would give a higher crossingover value than the later part in the first brood of a female which had been exposed to heat up to the time of hatching but mated at 22°. In other words, only part of the eggs in any brood may be affected by the temperature during the previous ten day period, and part of them by the temperature to which the female is exposed while laying that brood. Since any bottle is usually counted at about two day intervals, it is possible by

reference to the original records to set the count for approximately the first half of the brood against the second half for comparison. A few such fractionations taken at random gave the following results:

TABLE 12

S'

b	c
---	---

NUMBER	TOTAL	NON-CROSS-OVER	1	2	1-2	I	II
						<i>per cent</i>	<i>per cent</i>
Heat 31.5°, bred at 22°							
141, First count.....	207	93	50	45	19	33.0	30.8
141, First one-half.....	128	57	30	28	13	33.7	32.0
141, Second one-half.....	79	36	20	17	6	32.9	29.1
141, Second brood after 10 days 22°.....	302	164	91	35	12	34.1	15.6
145, First count.....	221	99	54	55	13	30.3	30.7
145, First one-half.....	149	67	29	41	12	22.5	35.5
145, Second one-half.....	72	32	25	14	1	36.1	20.8
145, Second brood after 10 days at 22°.....	195	110	53	20	12	33.3	16.4
Control 22°, bred at 22°							
150, First count.....	310	154	98	45	13	35.8	18.7
150, First one-half.....	213	103	66	32	12	36.0	20.0
150, Second one-half.....	97	51	32	13	1	34.0	14.4
150, Second brood after 10 days at 22°.....	364	207	107	40	10	32.1	13.7

In both of the bottles seeded by females which have been exposed to heat during their development, we find that the offspring hatched during approximately the first half of the period counted show a higher percentage of crossingover than those hatched later. The latter approach the value given by the second broods, which has already been shown to be practically the same as the second brood control value. Fractionation of a control culture shows a similar progressive decrease, though it is less striking because the difference between the first and second brood values is much less. Fractionation of the first ten day broods brings out then that the heat effect is more

striking than the actual counts registered in the first half of the next brood, but that it probably disappears entirely in the latter half. It also shows that the decrease between the first and second broods in the control series is a gradual one.

*Analysis of the temperature effect by counts at short intervals*

In view of this fact, it seemed reasonable to suppose that approximately the actual number of eggs which are affected by exposure to high temperatures for a given period could be determined if the output of a female could be counted for a series of intervals shorter than the usual ten day broods. In order to meet this difficulty, the following method was devised.  $F_1$  females from both control and heat-treated series were mated and placed in shell vials, 1 inch by 4 inches, with a small amount of food in the bottom. Each vial was marked with the serial number of the pair and the date when the pair was placed in it. These pairs were transferred to new vials after the females had been allowed to lay for two days, and such a series was continued as long as eggs were laid. The total for each two day interval of each series which had received the same treatment was taken, the percentage of crossingover calculated, and the average number of eggs hatched for the interval recorded. A table constructed on the basis of these results gives a complete picture of the variation in the percentage of crossingover throughout the entire output of any female. In the case of females which have been treated with high temperatures for a given period, it shows just how soon the effect of that temperature is reflected in an increase in crossingover, and just how long that effect persists. Between the control and treated series the crossingover percentages at any given point are comparable almost within the limits of the probable error, for they represent the mean of two series from the same  $P_1$  parent, run at the same time, and given the same food. In table 13 the results are given for three separate series, all from the same  $P_1$  parents, the second and third members of which received the heat treatment at different times. Care was not taken in this group to change the pairs



from one tube to another at exactly two day intervals in all cases. This introduced a slight element of uncertainty which was corrected in table 14. In this latter table a cold test was also run in order to give added confirmation to the results already reported. The two groups of data are as follows:

TABLE 13

b pr c

DATE	TOTAL NUMBER DAYS AFTER MATING	NUMBER OF DAYS IN TUBE	NUMBER OF TUBES	TOTAL	NON-CROSSOVER	1	2	1-2	I	II	AVERAGE PER TUBE
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Control Nos. 409-415. Hatched and mated at 22°C.

									per cent	per cent	
November 8.....	2	2	6	414	292	36	79	7	10.3	20.7	69
November 10.....	5	3	6	300	222	23	54	1	8.0	18.3	50
November 13.....	7	2	4	220	174	9	37	0	4.1	16.8	55
November 15.....	9	2	6	561	447	27	80	7	6.0	15.5	93
November 17.....	13	4	6	591	456	8	100	7	5.9	18.1	98
November 21.....	15	2	6	430	317	30	79	4	7.9	19.3	72
November 23.....	17	2	5	253	192	12	46	3	5.9	19.3	51
November 25.....	20	3	4	382	279	27	71	5	8.3	19.9	95
November 28.....	22	2	4	311	235	9	61	6	4.8	19.6	77
November 30.....	24	2	5	199	151	7	38	3	5.0	20.6	40
December 2.....	27	3	5	274	209	11	50	4	5.4	19.7	55
December 5.....	30	3	4	211	160	8	40	3	5.2	20.3	53
December 8.....	33	3	4	174	130	10	34	0	5.7	19.5	43

Nos. 402-407. Hatched at 22°C. Incubator at 31° from first to seventh day

November 6.....	7	7	6	1,714	1,249	135	302	28	9.5	19.2	284
November 13.....	9	2	4	207	130	28	39	10	18.3	23.7	52
November 15.....	15	6	6	416	256	54	85	21	18.0	25.5	69
November 17.....											
November 21.....	17	2	6	520	411	15	89	5	3.8	18.0	87
November 23.....	19	2	6	442	358	12	71	1	2.9	16.3	74
November 25.....	22	3	5	411	318	17	75	1	4.3	18.5	82
November 28.....	24	2	4	285	237	9	37	2	3.8	13.7	71
November 30.....	26	2	3	169	146	7	16	0	4.1	9.5	56
December 2.....	29	3	3	220	170	8	41	1	4.1	19.1	73
December 5.....	32	3	2	129	110	4	15	0	3.1	11.6	65
December 8.....	35	3	3	99	77	6	16	0	6.6	16.1	33

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TABLE 13—Continued

DATE	TOTAL NUMBER DAYS AFTER MATING	NUMBER OF DAYS IN TUBE	NUMBER OF TUBES	TOTAL	NON-CROSSOVER	1	2	1-2	I	II	AVERAGE PER TUBE
Hatched at 31°C., bred at 22°C. Nos. 428-437											
November 13.....	2	2	9	471	304	53	103	11	13.6	24.2	53
November 15.....	4	2	9	888	584	90	195	19	12.3	24.1	99
November 17.....	8	4	10	883	602	73	183	25	11.1	23.5	88
November 21.....	10	2	10	835	652	32	145	6	3.8	17.9	83
November 23.....	12	2	3	178	133	2	42	1	1.6	24.1	59
November 25.....	15	3	3	312	270	6	36	0	1.9	11.5	104
November 28.....	17	2	2	130	108	1	20	1	1.5	16.1	65
November 30.....	19	2	2	141	118	3	20	0	2.1	14.2	70
December 2.....	22	3	2	163	133	6	22	2	4.8	14.6	81
December 5.....	25	3	2	150	121	4	24	1	3.3	16.6	75
December 8.....	29	4	2	93	73	6	13	1	7.5	15.0	41
Returned to incubator on tenth day. Nos. 428, 429, 430, only											
November 23.....	15	5	3	561	481	12	67	1	2.3	11.9	187
November 28.....	22	7	3	318	227	30	54	7	11.6	19.1	106

The results given in these two tables are brought out much more clearly when put in the form of a curve. Such curves for both of the crossover regions involved are given in figures 4 to 8.

In all cases the horizontal series denotes successive days after mating, while in the vertical series are ranged the percentages of crossingover. In figures 4, 5 and 6 the results for both regions in each of the series which received the same treatment are shown in one figure. The correspondence in the form of the curves is seen to be close, except in the later portion where the numbers recorded are small. In figures 7 and 8 the values for the same region in each of the two series are set against each other. Each of these figures brings out what can be gained only by comparing figures 4, 5 and 6 with each other, namely, the

TABLE 14  
b p r c

DATE	TOTAL NUMBER OF DAYS AFTER MATING	NUMBER OF DAYS IN TUBE	NUMBER OF TUBES	TOTAL	NON-CROSSOVER	1	2	1-2	I	II	AVERAGE PER TUBE
Control. Hatched and bred at 22°C. Nos. 592-606											
February 7.....	3	3	13	1,289	926	86	256	21	8.3	21.5	99
February 10.....	5	2	13	1,694	1,299	67	313	15	4.9	19.4	130
February 12.....	7	2	13	1,393	1,046	74	252	21	6.8	19.6	107
February 14.....	9	2	13	1,399	1,062	64	257	16	5.8	19.5	108
February 16.....	11	2	12	1,212	946	41	215	10	4.2	18.6	101
February 18.....	13	2	11	730	551	31	142	6	5.1	20.3	67
February 20.....	15	2	11	1,110	864	44	187	15	5.3	18.2	101
February 22.....	17	2	11	946	757	34	149	6	4.2	16.4	86
February 24.....	19	2	11	1,193	899	72	207	15	7.3	18.6	108
February 26.....	21	2	9	828	599	53	161	15	8.2	21.3	92
February 28.....	23	2	3	302	221	21	57	3	7.9	19.8	101
March 2.....	25	2	4	256	186	15	51	4	7.0	21.1	64
March 4.....	28	3	3	135	102	7	25	1	5.9	19.2	45
March 7.....	31	3	2	211	174	4	33	0	1.9	15.6	105
March 10.....	33	2	2	110	86	3	19	2	4.5	18.2	55
March 12.....	36	3	2	160	119	4	35	2	3.7	23.4	80
March 15.....	38	2	2	146	115	6	24	1	4.8	17.1	73
March 17.....	40	2									
March 19.....	42	2									
March 21.....	44	2	1	71	57	2	12	0	2.8	16.9	
Cold 9°C. Nos. 592, 593, 595 and 596, 597, 598, placed in refrigerator from the twenty-first to the twenty-eighth day after mating											
February 28.....	23	2	6	No eggs laid							
March 2.....	25	2	6	No eggs laid							
March 4.....	28	3	6	No eggs laid							
March 7.....	31	3	4	173	127	8	33	5	7.5	21.9	43
March 10.....	33	2	4	323	219	21	70	13	10.5	25.7	81
March 12.....	36	3	4	316	203	30	69	14	13.9	26.2	79
March 15.....	38	2	4	356	221	44	79	12	15.4	25.3	89
March 17.....	40	2	4	241	172	20	42	7	11.2	20.3	60
March 19.....	42	2	4	175	119	11	42	3	8.0	25.6	44
March 21.....	44	2	3	253	179	17	50	7	9.5	22.5	84
March 23.....	47	3	3	222	154	21	41	6	12.2	22.2	74
March 26.....	50	3	2	129	101	2	26	0	1.5	20.2	65

TABLE 14—*Continued*

DATE	TOTAL NUMBER OF DAYS AFTER MATING	NUMBER OF DAYS IN TUBE	NUMBER OF TUBES	TOTAL	NON-CROSSOVER	1	2	1-2	I	II	AVERAGE PER TUBE
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Hatched at 22°C. Exposed to 31.5°C. from third to eleventh day after mating (also from nineteenth to twenty-first day). Nos. 607-626

									per cent	per cent	
February 7.....	3	3	15	1,401	1,020	78	281	22	7.1	21.6	93
February 10.....	5	2	12	566	419	19	120	8	4.8	22.6	47
February 12.....	7	2	12	781	602	26	150	3	3.8	19.6	65
February 14.....	9	2	13	660	525	19	110	6	3.8	17.6	52
February 16.....	11	2	13	428	306	33	84	5	8.8	20.8	33
February 18.....	13	2	12	466	302	50	99	15	13.9	24.5	39
February 20.....	15	2	11	697	385	92	178	42	19.2	31.6	63
February 22.....	17	2	10	558	310	80	136	32	20.0	30.1	56
February 24.....	19	2	9	598	345	85	146	22	17.5	28.9	66
February 26.....	21	2	7	235	169	10	50	6	6.8	23.8	23
February 28.....	23	2	7	385	292	18	74	1	4.9	19.5	55
March 2.....	25	2	4	219	178	3	38	0	1.4	17.3	55
March 4.....	27	2	4	167	120	7	39	1	4.8	23.9	42
March 6.....	29	2	3	150	112	12	26	0	8.0	17.3	50
March 8.....	31	2	3								
March 10.....	33	2	3	207	169	2	35	1	1.4	17.4	69
March 12.....	35	2	3	192	146	6	37	3	4.6	20.8	64
March 15.....	37	2	2								
March 17.....	39	2	1	93	75	2	16	0	2.1	17.1	

No. 626. Not added to above. No eggs hatched from February 18 to February 26, inclusive, new male February 28

February 28.....	23	2	1								
March 2.....	25	2	1	132	76	23	25	8	23.4	25.0	
March 4.....	27	2	1								
March 6.....	29	2	1	84	69	3	12	0	3.5	14.3	

difference in the form of the curves caused by the application of heat or cold.

*Interpretation of the tube data*

*Control 'curve of age.'* The conclusions to be drawn from the data given in tables 13 and 14, and from its graphic presentation in figures 4 to 8 can be stated briefly. First, with regard to the percentage of crossingover in the control, our results as given in figure 4, and for a longer period in figures 7 and 8 show beyond

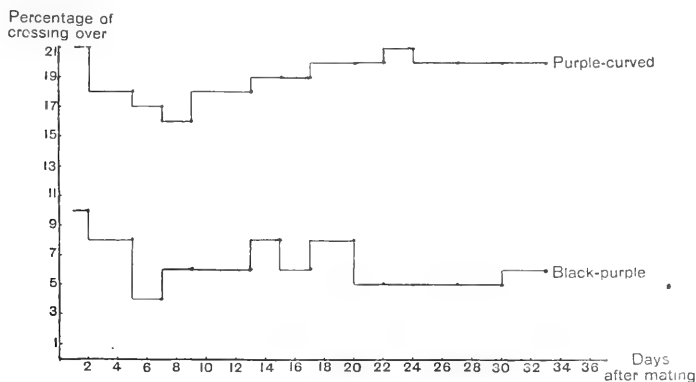


Fig. 4. Control values for both regions from table 13

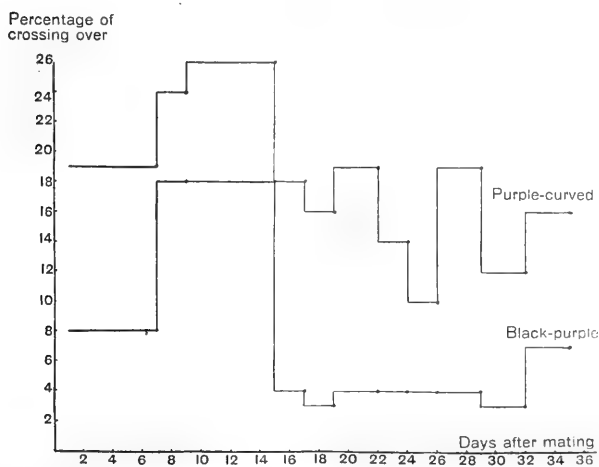


Fig. 5. Curves of percentage of crossingover for both regions shown by the offspring of females hatched at 22° and exposed to 31.5° for the first seven days after hatching (table 13).

question that this value varies between a maximum and a minimum separated by six or seven units, and that this variation has a perfectly definite rhythm. The control value always starts

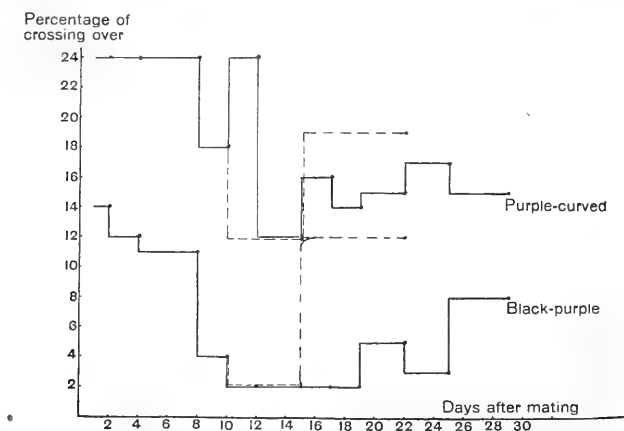


Fig. 6. Curves of percentage of crossover for both regions shown by the offspring of females hatched at  $31.5^{\circ}$  but mated at  $22^{\circ}$ . Broken line indicates percentages from three pairs returned to the high temperature on the tenth day (table 13).

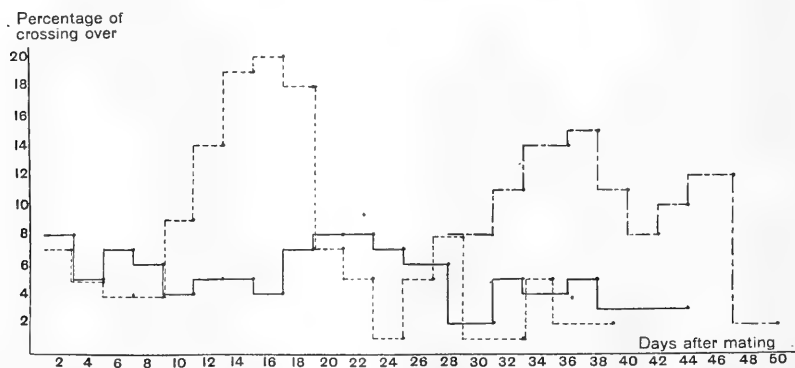


Fig. 7. Curves of percentage of crossover for the black-purple region from table 14. — = control-hatched and mated at  $22^{\circ}$ ; - - - = hatched at  $22^{\circ}$ —exposed to  $31.5^{\circ}$  from the third to the eleventh day. — — — = six control pairs exposed to  $9^{\circ}$  from the twenty-first to the twenty-eighth day (no eggs laid during the interval).

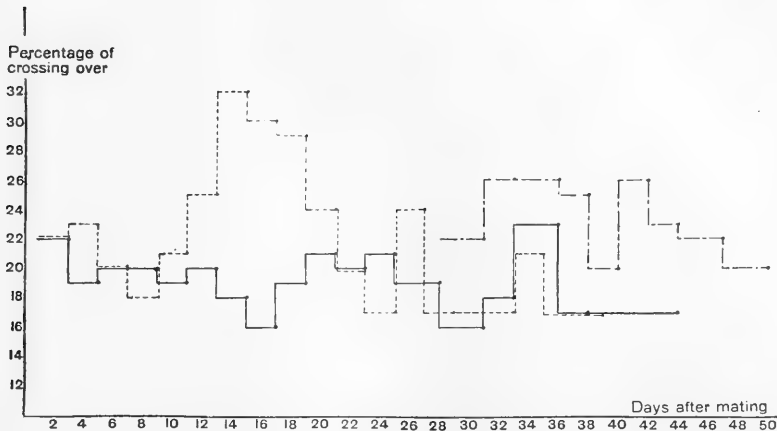


Fig. 8. Curves of percentage of crossingover for the purple-curved-region from table 14. (Legend as in figure 7.)

rather high, and drops gradually to a low point which is reached between the 7th and the 10th day. It remains at close to this point until about the sixteenth day, when a rise begins, which brings it up again to the maximum after about twenty-two to twenty-four days. A second drop brings the value down again before the thirtieth day. In the longer curves there is even a suggestion of a second rise, but after about the thirtieth day the numbers are too small to be of great significance. By cutting such a curve into ten day periods a series of percentages can be secured which corresponds to those recorded by Bridges, and confirmed in this paper. The first ten day brood catches the initial high point and the first drop; the second brood, the minimum and the beginning of the first rise, while the third ten day period includes the second high point and the second drop. The first brood would give the highest value, the third the next highest (if the second maximum is not quite as high as the initial one) and the second the lowest. This 'curve of age' must be intimately connected with certain physiological processes in the body of the female which have some 'environmental' effect on the developing eggs. The first drop in the curve might be explained by the gradual change through which the body goes during the metamorphosis, when the eggs which register the

drop are formed, but such a suggestion does not account for the later behavior of the curve.

*Heat effect at one stage in the ovary only*

The data given in the two day interval series with regard to the effect of heat on crossingover is happily very much more striking, and extremely easy to interpret. Figure 5 gives the analysis of the offspring of flies which were hatched from the same bottles as those which made up the control series. The heat was applied immediately after mating and was continued for seven days. The flies were kept in the same tubes throughout this period. The percentage of crossingover indicated is that of the initial high point of the control. The next count taken represents the total for the period from the seventh to the ninth day, and the results are 9 and 5 units higher than the previous values respectively, and 12 and 9 units higher than the corresponding control values. This high percentage is maintained for eight days at the end of which the curves drop abruptly 14 and 7 units respectively, and at the end of the seventeenth day show values even below the corresponding ones for the control. This low level is then maintained throughout the series.

Figure 6 gives a similar picture as a result of slightly different treatment. The  $F_1$  females of this series were hatched at  $31.5^{\circ}\text{C}$ . but mated and kept at  $22^{\circ}\text{C}$ . The percentages of crossingover begin at a point 4 to 5 units above the control and this high level is approximately maintained for eight days. The curve then drops to the normal point and stays there throughout the remainder of the series. (The exception in the ten to twelve day value for the purple-curved region apparently is insignificant statistically, and especially so when viewed in contrast to the corresponding black-purple value.) At the tenth day three of the pairs were again subjected to a temperature of  $31.5^{\circ}\text{C}$ ., and the results are shown by the broken line. The same values given by the untreated pairs are maintained for five days, at the end of which the curve jumps a second time to the original heat-induced point.



Figures 7 and 8 show the same facts with larger numbers and a value for each two day period. The broken line shows the values for the offspring of females hatched as in figure 5 from the same cultures as the controls. They were mated and kept for three days at 22°C. At this point they were exposed to a temperature of 31.5°C. which was continued for eight days. The curve continues at approximately the same level as the control for eight days, and then rises in two distinct steps. This high point is maintained for about eight days when the curve comes down with considerable abruptness to about the control value, where it remains.<sup>2</sup> At the twenty-first day the control series was divided and six tubes were subjected to a cold test of 9°C. for seven days. During this period no eggs were laid and therefore no results are recorded. On the thirty-first day, however, a rise was begun and the curve was maintained at a point well above the control for seven days. Then a drop occurred, followed by a second but smaller rise, after which the control value was again reached. This last rise would seem to mean that when as low a temperature as 9°C. is used a slight heat stimulation results by transferring the flies back to normal temperature.

The facts which these tables and curves bring out are briefly these. Exposure of a female to a temperature of 31.5°C. for a given period results in a decided increase in the percentage of crossingover among her offspring for a period about as long as the exposure. In case she is exposed up to the time of hatching this increase shows among the offspring of the first seven or eight days after mating. If the adults are exposed, the effect begins to be noted at the seventh or eighth day after the adults first began to receive the treatment. The increase is maintained for the definite period mentioned above only. When the value has again returned to that of the control no further deviation is observable, though the rhythm of the 'curve of age' seems to have been disturbed. The facts admit of only one interpreta-

<sup>2</sup> This series was subjected to a second exposure to 31.5°C. from the 21st to the 23d day. A slight rise was recorded in the curve about the 27th day. The failure to show the usual maximum for an exposure of only two days is discussed in a later section.

tion, namely, that the high or low temperature has an effect on the chromosomal mechanism at one point in the development of the eggs only, and that eggs which have already passed this particular point in their development when the new temperature is applied, or which do not reach that point during the period of exposure, register no higher percentage of crossingover than the controls.

#### VI. LOCALIZATION OF PROBABLE POINT OF CROSSINGOVER IN OVARIES

##### *Genetic data*

From the data given in tables 13 and 14 it is possible to locate the point where this effect is produced with fair numerical exactness. One has only to add the sums of the average tube counts for the periods from the time that heat was applied to the time when the first significant rise in the curve takes place. The same total ought to be given by adding the sums of the periods between the time when the exposure is discontinued to the time when the drop to the control value occurs. Such an operation gives the following results:

TABLE 15

NUMBER		NUMBER OF FLIES HATCHED BEFORE EFFECT IS SHOWN	NUMBER OF FLIES HATCHED BEFORE EFFECT DIS- APPEARS
1	Figure 5, table 13.....	284	121
2	Figure 6, table 13: first drop.....		240
3	second rise.....	187	
4	Figure 7 and 8, table 14.....	197	224

A glance at the tables shows that some of these figures must be smoothed considerably. In figure 5 only one count was taken for the first seven days. It seems certain that if this period had been broken up the rise would have begun sooner, and the total of 284 would have been much less. The same condition obtained to a less degree with the second total—240—which is probably not much too high. The very low value of

TABLE 16  
b p r c

DATE	TOTAL NUMBER OF DAYS AFTER MATING	NUMBER OF DAYS IN TUBE	NUMBER OF TUBES	TOTAL	NON-CROSSOVER	1	2	1-2	I	II	AVERAGE PER TUBE
------	---	---------------------------	--------------------	-------	---------------	---	---	-----	---	----	---------------------

Control, hatched and mated at 22°C. Nos. 660-664

									per cent	per cent	
March 29.....	2	2	5	241	165	16	55	5	8.6	24.9	48
March 31.....	4	2	5	543	415	26	100	2	5.1	18.8	109
April 2.....	6	2	5	564	430	16	116	2	3.2	20.8	113
April 4.....	9	3	5	538	426	17	94	1	3.3	17.6	108
April 7.....	11	2	5	418	326	22	70	0	5.2	16.7	84
April 9.....	13	2	5	259	184	21	51	3	9.0	20.8	52
April 11.....	17	4	4	252	198	10	42	2	4.7	17.4	50

Hatched, 22°C. Exposed to 31.5°C. from second to fourth day after mating.  
Nos. 665-669

March 29.....	2	2	5	212	157	12	39	4	7.5	20.3	42
March 31.....	4	2	4	300	222	14	63	1	5.0	21.3	75
April 2.....	6	2	3	261	202	8	51	0	3.1	19.6	87
April 4.....	9	3	3	383	309	14	57	3	4.4	15.6	103
April 7.....	11	2	3	224	130	18	71	5	10.2	33.9	43
April 9.....	13	2	3	136	98	6	30	2	5.9	23.5	33
April 11.....	17	4	3	178	132	8	40	0	4.4	22.3	44

Hatched, 22°C. Exposed to 31.5°C. from second to sixth day after mating.  
Nos. 675-679

March 29.....	2	2	5	188	145	9	34	0	4.8	18.1	38
March 31.....	4	2	4	248	190	5	51	2	2.8	21.4	62
April 2.....	6	2	3	130	99	4	25	2	4.5	20.7	43
April 4.....	9	3	3	247	183	22	37	5	10.9	17.0	82
April 7.....	11	2	2	156	98	17	35	6	14.7	26.3	78
April 9.....	13	2	2	136	84	18	33	1	13.9	25.0	68
April 11.....	17	4	4	273	183	25	60	5	11.0	23.7	68

121 for the disappearance of the effect after heat was removed in figure 5 results from the fact that one two day count (November 17 in table 13) failed entirely, resulting in a loss of about one hundred flies from the total. The items in the first column of Nos. 3 and 4 (table 15) are probably slightly lower than the actual num-

ber because the viability of eggs laid at  $31.5^{\circ}\text{C}$ . is rather high. The value in the second column of No. 4 is open to none of these criticisms, and is approached by all the other values when proper allowance is made for them. On the basis of the data in hand, then, and taking into account the ordinary viability<sup>3</sup> one would expect that in the ovaries of females not more than ten days after mating temperature produces a measurable effect at a stage so situated that 225 to 275 eggs may be laid before the eggs which have passed through that stage can be laid. This rather involved statement will be made clearer in connection with cytological evidence which is given below.

Before considering this evidence it becomes necessary to speak of some further genetic data which has a direct bearing on the statement made above. We have so far proceeded on the assumption that high temperature becomes immediately effective in changing the percentage of crossingover as soon as applied. A series of fifteen pairs were made up exactly as in table 14—hatched from the same culture at  $22^{\circ}\text{C}$ . Five of these were continued at  $22^{\circ}\text{C}$ . as a control, five were subjected to a temperature of  $31.5^{\circ}\text{C}$ . for two days, and five for four days. The results are given in table 16 and the percentages of crossingover for the black-purple region are plotted in figure 9. The rise recorded by the offspring of the series exposed for only two days is less than half as high as is that reached by the series exposed for four days during the same two day interval. It is not greatly above the high point of the control series. The four day exposure results in a rise in the percentage of crossingover to the same point reached as a result of an exposure for eight days, and there it remains for slightly more than four days' time. If the temperature began to affect the developing eggs as soon as applied, the two day exposure should have caused a rise to the same maximum for a period of two days. Since the curve shows that only a few eggs were affected, it appears that an exposure of at least two consecutive days to a temperature of  $31.5^{\circ}\text{C}$ .

<sup>3</sup> R. C. Hyde (*Jour. Exp. Zool.*, 1914, vol. 17, p. 369) considers a fertility of 83.6 per cent (total number of flies hatched divided by the number of eggs laid) as unusually high for fertile stocks.

is necessary to produce the usual effect on the percentage of crossingover. Yet when the high point is reached it is maintained for approximately the same length of time as the exposure, and not for two days less. The same two day period must elapse, therefore, before the effect of the return to room temperature is registered by a change in the amount of crossingover. This means that what may be termed an 'incubation period' of slightly less than two days' consecutive exposure to the new temperature is necessary before the effect on the developing eggs is produced.

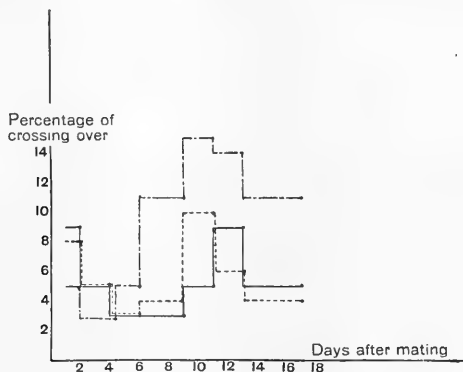


Fig. 9. Curves of percentage of crossingover for the black-purple region from table 16. — = control-hatched and mated at 22°; ---- = hatched at 22° exposed to 31.5° from the second to the fourth day; — — — = hatched at 22° exposed to 31.5° from the second to the sixth day.

Reference to table 16 shows that the average number of flies hatched during such a period is over a hundred, and perhaps one-third more eggs are laid but do not hatch. It is certain, then, that at least one hundred eggs are normally laid during the 'incubation period,' and therefore that in any calculation such as that given above we must subtract the total number of eggs which would be unaffected because of the existence of this 'incubation period.' The corrected value is, then, 125 to 175 eggs. Eggs in such a stage in the ovary that 125 to 175 eggs will be laid before them are without much question in the stage at which a change in the percentage of crossingover due to heat is brought about.

With the final statement of this result the implication of the discussion becomes obvious. High temperature causes a definite change in the amount of crossingover. If the point where this change occurs in the ovary can be established, there is strong evidence for the belief that that is the point where crossingover normally occurs. An examination of the ovaries of females soon after hatching therefore becomes extremely important.

### *Cytological data*

For a considerable period the author in collaboration with Dr. C. W. Metz has been endeavoring to work out the cytological features of *Drosophila melanogaster* (*seu* *ampelophila*). A considerable amount of material was therefore at hand. Sections of the whole abdomens of females about twelve hours old were selected, and drawings were made of all of the sections of one ovary from each of two different females. Each ovary was made up of five or more egg strings, as is common in insects, and the eggs go through the growth period as they pass from one end to the other. This results in a definite seriation from oogonia at one end to large, mature eggs ready for fertilization at the other. Because of the fact that oogonia are not surrounded by an envelope of follicle cells,<sup>4</sup> it was an easy matter to mark the cysts of oogonia with a cross as the drawings were made. When each of the series was complete the cysts which were not crossed were all oocytes. Each cyst is surrounded by a follicular envelope and contains sixteen nuclei, one of which is the future egg nucleus and the other fifteen are nurse cells. Beginning with the drawing of the first section each cyst could be traced individually throughout each of the succeeding sections in which it appeared. And all except the first were checked. When this process was carried through the entire series the total number of cysts or eggs which remained blank represented the total number of oocytes in the ovary. The totals recorded in these counts which include, of course, oocytes just past the last oogo-

<sup>4</sup> . . . and for other reasons which will be discussed in a later paper on the oogenesis in this fly.

nial division with the follicular envelope just forming up to the large mature eggs ready to pass down the oviduct, were 69 and 70. The two females would have in two ovaries of this size 138 and 140 oocytes respectively. The correspondence between these actual oocytes present and the numbers given above calculated from the genetic evidence is exact, and practically establishes the conclusion, that the change in the percentage of crossingover, and therefore without much doubt the crossingover process itself, takes place in the very early oocytes just after the last oogonial division. The cytological picture at this stage does not properly belong in this paper, but it will be of interest to say that at this point the nuclei of the nurse cells and the egg proper are indistinguishable, and the chromosomes in all are in what resembles a late leptotene or early diplotene condition in other forms.

#### *Corroborative evidence*

*Exceptional tube counts.* What remains to be said is simply corroborative of the above conclusion. If the crossingover process is determined at this early stage, and if for any reason the eggs are not laid continuously after the heat has been withdrawn, but after the high point has been registered by previous counts, then the high percentage should be maintained when the process of laying is begun again. Such a case occurred in the series recorded in table 14. Female No. 626 had been exposed to heat with the other pairs in the series and the percentage of crossingover among her offspring laid between February 16 and 18 showed, like the others, a slight increase. Between February 18 and 28 no eggs hatched in vials 626. On February 28 the record shows that the female was served with a new male, and from that point on offspring appeared as before. Because of the interruption, caused perhaps by the sterility of the previous male, the results were not added in with the remainder of the series. When the percentages of crossingover among her offspring laid between February 28 and March 2 were figured, it was found, table 14, that they showed the high value, although the remainder of the series had been giving the control value for

six days. The next total, March 2 to 6, gave the control value, showing that all of the eggs which had been affected by the high temperature had finally been laid.

*Ineffectiveness of exposure of early larvae.* A second set of data gives very strong corroborative evidence. In the developing female gonads very few oocytes are formed until the later larval and pupal condition is reached. If heat affects only the early oocytes, we should expect little or no increase among the offspring of females which had been exposed only in the early larval condition, and conversely practically the whole increase among offspring of females which had been exposed in the period from the late larval to the adult stage. Cultures were exposed to heat for four days after the eggs were laid, and then removed to normal temperature. Similar cultures were kept at 22° for the first four days and then transferred to 31.5°C. until the flies hatched. The test of these females compared with their corresponding controls follows:

TABLE 17

$$\frac{S'}{b \quad c}$$

NUMBER	TOTAL	NON-CROSS-OVER	1	2	1-2	I	II
						<i>per cent</i>	<i>per cent</i>
1, 4 days at 22°—6 days at 31.5°C..	1,851	855	524	329	143	36.0	25.5
2, control.....	822	425	245	110	42	35.0	18.5
3, 4 days at 31.5°—6 days at 22°....	1,519	758	442	228	91	35.0	21.0
4, control.....	1,195	579	371	184	61	36.1	20.5

The result shows that the expectation is justified, for there is practically no increase of No. 3 over No. 4, while the per cent II value for No. 1 is 7 units higher than No. 2 and is close to the usual value shown when the females are exposed for the whole period. The value given in No. 3 is more striking when compared with the value for four days' exposure in table 15, for the latter shows that such an exposure of adult flies is sufficient to raise the value to the high point for an equal period.



*Discussion of data*

*Synaptic stage probable point of crossingover.* In their Mechanism of Mendelian Heredity, Morgan, Sturtevant, Muller and Bridges give on page 131 a full discussion of the possible stages in oogenesis at which crossingover may occur. These authors show that an interchange between homologous chromosomes may take place either at an early stage in the growth period when the chromosomes are fine threads, or at a late stage when they are thicker and shorter. The second point was the one taken by Janssens as the basis for his chiasmatype theory. The first named authors do not suggest which stage appears to be the more probable point of crossingover. Later Muller ('16) concluded that the fine thread stage appeared to be the more likely point, and recently Bridges has inclined to the same conclusion. By analogy with other forms the stage which formed the basis of Janssens' chiasmatype hypothesis—the diakinesis, as it is often called—must occur after the egg has completed its growth and is practically ready for fertilization, which undoubtedly precedes polar body formation. If the crossingover occurred at this point any change caused by temperature would become evident at once (or, allowing the same 'incubation period,' after about 100 eggs had been laid) in the percentages of crossingover shown by the offspring, for there are not more than ten eggs in the two ovaries which are in this stage or beyond. This point taken in conjunction with the positive evidence from the counts given in this paper makes it highly probable that crossingover occurs in the very early growth period, when the chromosomes are known to be in the form of thin threads, and at that point only.

*Disproof of reduplication hypothesis.* A word must be said regarding the reduplication hypothesis. This interpretation of linkage by Bateson and his followers involved a set of divisions at which certain factors were segregated, resulting in a set of cells which developed at unequal rates. Sturtevant has already shown that the theory is a mathematical absurdity when applied to a set of linked factors in *Drosophila*. It is evident that the experimental data given in this paper absolutely disprove the

hypothesis. It has been shown positively that crossingover does not occur in the early oogonial divisions, and there is good reason to believe that the percentage of crossingover is affected by temperature at one point in the growth period of the egg only. After this point, only the two polar body divisions take place, and at each of these divisions only one nucleus remains in the egg. The hypothesis cannot therefore explain crossingover in the second chromosome of *Drosophila*, and must be discarded as a serious attempt to account for the observed phenomena of linkage.

#### VII. FAILURE OF TEMPERATURE TO AFFECT CROSSINGOVER IN FIRST AND THIRD CHROMOSOME

In view of the very positive way in which temperature affects the percentage of crossingover in the second chromosome, it was natural to expect that a similar effect would be found to result from a similar treatment of females which were heterozygous for factors in the first and third chromosomes. Strangely enough, the tests so far made show that this is not the case. Using the sex-linked characters, vermilion eyes, sable body color gar-

TABLE 18\*

HATCHED AT	TOTAL	v s g f									
		1	2	3	1-2	1-3	2-3	1-2-3	I	II	III
									per cent	per cent	per cent
Control 22°.....	548	43	8	66					7.8	1.4	12.0
Heat, 32°.....	349	26	6	28		3			8.3	1.7	8.8
Second broods after 10 days at 22°.....	486	46	8	54					9.4	1.6	11.1
17.5°.....	2,736	285	36	349		3	2		10.5	1.4	12.9
Second broods after 10 days at 22°.....	1,015	77	13	109	1	1			7.6	1.4	8.4

v g s f											
Control 22°.....	1,420	110	25	165	1	1		2	8.0	1.9	11.1
Heat, 31.0°.....	516	38	5	50				2	7.7	1.3	10.1

\* To save space the non-crossover class is omitted from this table.

net eyes, and forked bristles—v-s-g-f—a series of tests was made which in every way resembled those made with second chromosome stock. These characters were used in two different combinations, as shown in the table. The results were as above.

The percentages of crossingover show no significant differences at any of the four different temperatures used. It is also apparent that no decrease of any significance is observable in the second brood values. This fact was noted by Bridges at the time when he recorded the decrease in the second brood values for the second chromosome.

The third chromosome data is too limited to record at present, but it so far has shown no increase in the amount of crossing-over due to temperature. This failure of temperature to cause a change in the percentage of crossingover is interesting, though it in no way invalidates the conclusions drawn from the second chromosome data. Taken in conjunction with the fact that no alteration in the percentages for second broods is found, it may mean that the units in the three groups as we are accustomed to calculate them, do not have the same significance, or that the sections of the real chromosomes, which we know as genetic chromosomes, do not correspond. Whatever is the meaning of this difference in reaction to temperature, it gives one added reason for believing that the 'genetic chromosomes' are discrete elements which differ among themselves, and retain this individuality from generation to generation.

#### VIII. ACKNOWLEDGMENTS

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#### IX. SUMMARY

The foregoing paper has brought out the following important facts:

1. Wet and dry food, starvation, increased fermentation of the food, and probably solutions of  $F_2Cl_3$  are ineffective in causing a change in the percentage of crossingover among the offspring of a female to which these environmental influences are applied.

2. A temperature of  $31^\circ C.$  and of  $13^\circ C.$  applied to female flies while they are undergoing development from egg to adult causes a decided increase in the percentage of crossingover among the offspring of the first brood, but not of the second brood.

3. Tests of offspring of females treated as in No. 2 above at an ascending series of temperatures gives a curve which rises from  $9^\circ C.$  to a first maximum of  $13^\circ C.$ , drops through  $17.5^\circ C.$  to a minimum extending from  $22^\circ C.$  to  $27^\circ C.$ , rises again through  $29^\circ C.$  to a still higher maximum at  $31^\circ C.$ , after which a slight fall is again noted. This indicates some sort of a change in the physical state in the structural makeup of the nuclear mechanism.

4. More detailed analysis shows that the high percentage of crossingover can be induced among the offspring by at least two consecutive days' exposure of the females to high temperature from their late larval period on. If the females are exposed before hatching the increase is noted among their first offspring, but if the exposure comes after the adult stage is reached the high percentage does not appear until between 225 and 275 eggs have been laid. The percentage of crossingover remains at the high point for approximately the same number of days that the female parent was exposed to the high temperature, and then drops to the control value.

5. The facts given in No. 4 are taken to indicate that the high temperature influences crossingover at one point in the oogenesis only. Since nearly two consecutive days' output of eggs is to be subtracted from the total given above, due to the existence of an 'incubation period,' we may consider a stage in oogenesis which is so situated that between 125 and 175 eggs in the ovary are more advanced than this stage, represents approximately the point at which crossingover takes place.

6. Actual examination shows that there are on an average 140 oocytes present in a female which is newly hatched. There is, therefore, good ground for the belief that crossingover occurs

in the very earliest oocytes. This is corroborated by the fact that high temperature has little effect if applied to females in the early larval condition.

7. This is strong evidence that crossingover does not occur at the late stage in oogenesis used by Janssens as the basis of the chiasmatype, and makes it probable that it occurs at the fine thread stage. The evidence also disproves the reduplication hypothesis as an explanation of linkage in *Drosophila*.

8. Tests of the first and third chromosomes by the same methods show that high and low temperatures are ineffective in causing a change in the percentage of crossingover. This is further evidence of the reality of the 'genetic chromosomes.'

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APPENDIX

DETAILED SUMMARIES OF INDIVIDUAL CULTURES  
ON WHICH TABLES IN THE TEXT  
ARE BASED

Tables numbered as in the text

TABLE 1

No. 1. See No. A—table 5

No. 2. See No. A—table 4

TABLE 2

*Effect of moisture in food on crossing-over***b pr c**

NUMBER	NORMAL	b pr c	pr c	b	c	b pr	b c	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
A. Control (usual amount of moisture)															
A I 2	75	71	5	7	13	32	4	1	208	146	12	45	5	per cent	per cent
3	115	75	5	6	25	27	3	2	258	190	11	52	5		
A II 1	102	81	7	7	23	30	1	5	256	183	14	53	6		
2	63	70	6	8	15	21	3	1	187	133	14	36	4		
4	120	123	11	7	30	37	2	2	332	243	18	67	4		
AIII 1	54	47	7	3	16	15	0	0	142	101	10	31	0		
2	43	57	5	4	17	19	2	2	149	100	9	36	4		
3	61	61	7	2	19	6	2	0	158	122	9	25	2		
Total.....									1,690	1,218	97	345	30	7.5	22.2
B. Very dry food															
AI 1	62	59	0	3	19	15	0	2	160	121	3	34	2		
B II 1	66	76	3	4	13	12	1	0	175	142	7	25	1		
2	52	62	5	5	20	23	0	2	169	114	10	43	2		
3	58	68	8	6	20	21	1	3	185	126	14	41	4		
4	84	85	8	11	21	14	1	1	225	169	19	35	2		
BIII 1	127	111	7	7	30	32	3	1	318	238	14	62	4		
2	61	58	6	2	18	19	1	2	167	119	8	37	3		
3	74	59	3	5	14	13	1	0	169	133	8	27	1		
4	42	61	6	4	14	17	3	3	150	103	10	31	6		
5	79	80	5	6	20	19	1	4	214	159	11	39	5		
Total.....									1,932	1,424	104	374	30	6.9	20.9

TABLE 2—*Concluded*

NUMBER	NORMAL	b pr c	pr c	b	c	b pr	b c	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
C. Very wet food															
C II 1	91	90	9	8	26	25	2	1	252	181	17	51	3		
2	52	57	3	3	11	15	0	1	142	109	6	26	1		
3	55	56	8	3	20	17	2	1	162	111	11	37	3		
4	63	66	7	2	16	16	0	1	171	129	9	32	1		
CIII 1	58	65	5	4	10	18	1	0	161	123	9	28	1		
2	48	36	4	3	13	14	4	2	124	84	7	27	6		
3	81	65	8	5	22	16	2	2	201	146	13	38	4		
4	60	46	8	6	10	22	1	2	155	106	14	32	3		
5	80	69	2	6	18	11	1	1	188	149	8	29	2		
6	51	46	5	1	16	17	0	1	137	97	6	33	1		
CIV 1	72	59	4	7	20	14	0	4	180	131	11	34	4		
2	84	79	4	3	27	24	2	3	226	163	7	51	5		
3	102	123	9	11	30	31	0	4	310	225	20	61	4		
4	103	101	5	9	24	31	3	0	276	204	14	55	3		
Total.....									2,685	1,958	152	534	41	7.2	21.5

TABLE 3

*Effect of starvation of parents on crossingover*

b pr c

NUMBER	NORMAL	b, pr	pr c	b	c	b pr	b c	pr	TOTAL	NON-CROSS- OVER	1	2	1-2	I	II
A. Control															
A1	65	62	2	3	17	24	0	0	173	127	5	41	0	per cent	per cent
2	31	39	7	7	8	11	0	0	103	70	14	19	0		
3	52	37	2	4	11	12	2	4	124	89	6	23	6		
4	77	59	6	1	17	18	1	0	179	136	7	35	1		
5	55	58	3	2	13	19	0	0	150	113	5	32	0		
Total.....									729	535	37	150	7	6.0	21.5
B. Parents starved															
B1	38	21	2	3	7	6	1	0	78	59	5	13	1		
2	59	72	4	0	18	17	0	1	171	131	4	35	1		
3	55	51	2	4	11	11	1	0	135	106	6	22	1		
4	40	50	3	5	10	13	1	0	122	90	8	23	1		
Total.....									506	386	23	93	4	5.3	19.1



TABLE 4

*Effect of dextrose in food on crossingover*

b pr c

NUMBER	NORMAL	b, pr c	pr, c	b	c	b, pr	b, c	pr	TOTAL	NON-CROSS- OVER	1	2	1-2	I	II
A. Control															
A I ①	102	86	2	5	21	28	0	1	245	188	7	49	1	03.2	20.2
②	124	110	2	7	16	18	0	0	277	234	9	34	0	03.2	12.3
③	101	98	5	5	24	27	1	5	266	199	10	51	6	06.0	21.4
4	118	98	6	7	24	24	3	1	281	216	13	48	4	06.0	18.5
A II 1	63	49	3	8	11	16	1	0	151	112	11	27	1	07.9	18.5
2	45	41	3	1	8	10	0	0	108	86	4	18	0	03.7	16.5
A III 1	82	94	2	0	16	13	1	0	208	176	2	29	1	01.4	14.5
2	69	58	3	4	23	26	1	0	184	127	7	49	1	04.3	27.2
3	94	63	2	0	14	16	1	0	190	157	2	30	1	01.6	16.3
BIR ①	93	93	0	3	10	24	2	1	226	186	3	34	3	02.6	16.4
2	91	112	12	3	15	13	1	2	249	203	15	28	3	07.2	12.5
3	106	37	5	1	26	10	0	0	185	143	6	36	0	03.2	19.5
4	107	95	3	0	14	18	1	1	239	202	3	32	2	02.1	14.2
BIIR 1	67	92	2	5	22	26	4	0	218	159	7	48	4	05.0	23.8
2	57	41	2	4	16	7	0	0	127	98	6	23	0	04.7	18.1
3	108	100	8	5	25	27	4	2	279	208	13	52	6	06.8	20.0
Total.....									3,433	2,694	118	588	33	04.4	18.2

## B. Food contained teaspoonful of c. p. dextrose

B I 1	100	92	2	5	19	11	0	0	229	192	7	30	0	03.0	13.1
2	112	96	2	3	10	18	1	2	244	208	5	28	3	03.3	12.7
4	124	106	2	1	17	18	1	0	269	230	3	35	1	01.5	13.4
B II 1	97	50	8	1	17	11	0	0	184	147	9	28	0	04.9	15.2
2	91	85	5	2	13	21	0	1	218	176	7	34	1	03.7	16.0
3	67	48	1	3	11	8	0	0	138	115	4	19	0	02.9	13.8
B III 1	92	84	4	4	18	21	0	1	224	176	8	39	1	04.0	17.8
2	70	68	3	0	14	14	1	1	171	138	3	28	2	02.9	17.5
AIR 2	90	89	2	2	19	17	2	0	221	179	4	36	2	02.7	17.1
3	79	68	8	10	19	30	0	4	218	147	18	49	4	10.0	24.2
4	95	86	11	8	30	25	3	3	261	181	19	55	6	09.6	23.4
A IIR 1	90	101	5	7	19	22	1	0	251	197	12	41	1	05.2	16.7
Total.....									2,628	2,086	99	422	21	04.5	16.8

TABLE 5

*Preliminary analysis of effect of temperature on crossingover*b pr c

NUMBER	NORMAL	b, pr, c	pr, c	b	c	b, pr	b, c	pr	TOTAL	NON-CROSS-OVER	1	2	1-2	I	II
A. Female parent hatched at 22°. Control															
8I ①	85	75	8	6	13	36	4	5	232	160	14	49		9 09.9	25.0
②	62	70	9	6	21	28	2	0	198	132	15	49		2 08.5	25.7
③	97	80	7	8	19	33	1	3	249	177	15	53		4 07.6	22.8
④	112	97	23	13	26	41	5	0	317	209	36	67		5 12.3	32.7
⑤	85	89	8	7	14	23	0	3	229	174	15	37		3 07.8	17.4
20I ①	91	67	6	2	15	21	0	2	204	158	8	36		2 04.9	18.4
②	91	75	6	2	18	19	2	1	214	166	8	37		3 05.1	18.3
③	100	54	6	4	17	22	1	2	206	154	10	39		3 06.3	20.3
21I ①	101	108	7	5	18	24	1	0	264	209	12	42		1 04.9	16.2
③	81	74	8	5	19	20	0	1	208	155	13	39		1 06.7	19.5
22I ①	105	91	8	7	25	25	0	3	264	196	15	50		3 06.8	20.0
②	103	98	7	3	17	24	1	0	253	201	10	41		1 04.3	16.6
③	90	80	8	6	22	25	1	0	232	170	14	47		1 06.4	20.7
23I ①	95	44	13	1	18	16	0	3	190	139	14	34		3 08.9	19.4
③	79	64	16	7	25	17	3	6	218	144	23	42		9 14.6	23.4
VI ①	70	41	5	4	10	13	1	0	144	111	9	23		1 06.9	16.6
Total.....									3,622	2,655	231	685		51 07.8	20.2

## B. Female parent hatched at 31°C.

8III ①	100	57	3	14	21	19	9	2	225	157	17	40	11	12.4	22.6
②	95	61	4	3	15	32	3	4	217	156	7	47		7 06.2	24.0
③	81	81	10	3	18	24	4	2	223	162	13	42		6 08.7	22.1
④	85	48	6	10	15	9	11	1	185	133	16	24		12 15.1	19.4
⑤	54	32	5	6	14	10	4	2	127	86	11	24		6 13.3	23.3
⑥	83	48	12	12	26	17	8	5	211	131	24	43		13 17.5	26.5
20III ①	76	43	6	10	23	16	3	4	181	119	16	39		7 12.2	25.4
③	65	42	11	12	23	18	5	2	178	107	23	41		7 16.8	26.9
21III ①	76	59	8	16	16	32	5	2	214	135	24	48		7 14.5	25.7
②	42	41	5	4	16	12	6	1	127	83	9	28		7 12.6	27.5
③	93	66	13	17	28	32	7	3	259	159	30	60		10 15.4	27.0
22III ①	36	29	5	2	13	11	4	0	100	65	7	24		4 11.0	28.0
②	93	66	12	14	28	31	7	2	253	159	26	59		9 13.8	26.8
③	60	31	9	10	34	22	7	4	177	91	19	56		11 16.9	37.8

TABLE 5—Continued

NUMBER	NORMAL	b, pr, c	pr, c	b	c	b, pr	b, c	pr	TOTAL	NON-CROSS- OVER	1	2	1-2	I	II
														per cent	per cent
23III ①	76	61	13	17	26	32	11	5	241	137	30	58	16	19.0	30.6
②	58	58	6	10	20	14	6	1	173	116	16	34	7	13.3	23.6
③	75	28	10	5	20	15	6	2	161	103	15	35	8	14.2	26.7
VIII ①	93	73	15	15	54	29	11	5	295	166	30	83	16	15.6	33.5
Total.....									3,547	2,265	333	785	164	14.0	26.7

## C. Female parents hatched at 13°C.

8II ①	65	57	10	13	20	23	2	4	194	122	23	43	6	14.9	25.2
②	72	66	17	12	27	41	2	2	239	138	29	68	4	13.8	30.1
③	28	14	7	3	11	10	1	1	75	42	10	21	2	15.0	32.6
20II ①	36	34	4	10	12	13	7	3	119	70	14	25	10	20.1	29.3
②	72	67	13	16	35	32	1	5	241	139	29	67	6	14.5	30.2
③	103	89	12	17	35	34	5	4	299	192	29	69	9	12.7	26.0
21II ①	59	64	13	11	37	29	3	6	222	123	24	66	9	14.8	33.7
②	64	52	10	3	20	15	3	3	170	116	13	35	6	11.2	24.1
③	68	66	14	12	20	35	5	2	222	134	26	55	7	14.8	27.9
22II ①	50	46	4	6	19	17	3	2	147	96	10	36	5	10.2	27.9
②	80	96	16	11	22	31	1	7	264	176	27	53	8	13.2	23.1
23II ①	48	43	13	9	15	27	2	1	158	91	22	42	3	15.8	28.4
②	69	67	7	9	26	30	2	1	211	136	16	56	3	9.0	26.5
③	76	40	10	10	14	20	3	5	178	116	20	34	8	15.7	23.6
VII ①	94	69	8	10	16	30	3	3	233	163	18	46	6	10.3	22.3
Total.....									2,972	1,854	310	716	92	13.5	27.2

TABLE 6

*Further analysis of effect of temperature on crossingover*

b pr vg

NUMBER	NORMAL	b, pr vg	b	pr vg	c pr	vg	b, vg	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
A. Control, female parents hatched at 22°															
														per cent	per cent
648	126	116	10	13	15	30	3	3	316	242	23	45	6	9.1	16.1
649	156	166	16	9	34	28	2	3	414	322	25	62	5	7.2	16.1
650	135	86	12	16	7	18	3	2	279	221	28	25	5	11.8	10.7
653	79	86	16	7	13	16	2	0	219	165	23	29	2	11.4	14.0
654	144	104	17	10	24	23	1	5	328	248	27	47	6	10.0	16.1
656	129	70	18	16	19	22	0	4	278	199	34	41	4	13.6	16.2
657	122	113	12	12	27	17	1	1	305	235	24	44	2	8.5	15.0
Total.....									2,139	1,632	184	293	30	10.0	15.1
B. Female parents hatched at 31.5°C.															
639	101	108	13	14	21	19	0	7	283	209	27	40	7	12.0	16.6
643	97	72	13	12	22	14	4	6	240	169	25	36	10	14.6	19.1
645	81	90	10	23	22	29	7	6	268	171	33	51	13	17.1	23.8
646	100	101	22	20	32	24	5	4	308	201	42	56	9	16.8	21.1
Total.....									1,099	750	127	183	39	15.1	20.2

TABLE 7

*Further analysis of effect of temperature on crossingover*

$\frac{S'}{b \quad c}$																
NUMBER	S'	b, c	S', b, c	NOR- MAL	S' c	b	S' b	c	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II	
Female parents hatched at 22°C. Control																
D	1	32	38	18	22	14	9	5	5	143	70	40	23	10	35.0	23.3
	3	25	33	25	29	3	13	4	6	139	58	54	17	10	46.0	19.4
	4	37	52	26	34	23	12	7	5	196	89	60	35	12	36.7	23.9
	5	24	27	30	19	9	14	8	9	140	51	49	23	17	47.1	28.4
	6	31	34	31	26	20	12	11	5	170	65	57	32	16	42.9	28.2
	7	42	67	36	40	25	10	9	9	238	109	76	35	18	39.0	22.2
	9	29	28	30	22	12	6	6	11	144	57	52	18	17	47.9	24.3
	10	34	30	22	20	9	8	6	6	135	64	42	17	12	40.0	21.4
	11	46	37	35	24	12	10	7	4	175	83	59	22	11	40.0	18.8
	12	33	39	26	31	13	5	5	2	154	72	57	18	7	42.2	16.2
	13	29	36	24	17	10	6	5	6	133	65	41	16	11	39.0	20.1
	14	37	46	43	20	17	7	7	9	186	83	63	24	16	42.4	21.5
	15	18	46	52	15	9	11	10	3	164	64	67	20	13	48.7	20.1
F	1	62	60	30	39	3	18	3	9	224	122	69	21	12	36.2	14.7
	3	53	49	29	32	10	16	5	2	196	102	61	26	7	34.7	16.8
	4	40	50	28	24	9	17	7	1	176	90	52	26	8	34.1	19.3
	5	52	50	26	21	21	11	5	8	194	102 <sup>b</sup>	47	32	13	30.9	23.2
	8	44	37	26	30	10	11	4	5	167	81	56	21	9	38.9	17.9
	9	44	52	47	40	24	17	8	7	239	96	87	41	15	42.6	23.4
	10	44	45	34	31	9	17	8	5	193	89	65	26	13	40.5	20.2
	14	48	48	44	26	13	11	6	5	201	96	70	24	11	40.3	17.4
	11	37	46	25	23	12	12	10	5	171	83	48	24	16	37.4	23.4
	12	17	45	19	9	6	8	4	6	124	72	28	14	10	30.6	19.3
	13	30	46	32	18	9	12	6	1	156	76	52	21	7	37.8	17.9
	18	65	66	26	53	15	31	7	7	270	131	79	46	14	34.5	22.2
	19	52	52	31	44	17	30	9	10	245	104	75	47	19	38.4	26.9
	20	38	36	20	18	9	10	4	6	141	74	38	19	10	34.0	20.5
	147	68	96	31	40	17	27	5	6	290	164	71	44	11	27.8	19.0
	148	72	63	44	52	14	35	10	9	299	135	96	49	19	38.4	22.7
	150	72	82	63	35	16	29	6	7	310	154	98	45	13	35.8	18.7
	151	64	62	51	55	22	24	9	9	296	126	106	46	18	38.0	21.6
Total.....									6,009	2,827	1,915	872	395	38.4	21.1	

TABLE 7—Continued

NUMBER	S'	b, c	S', b, c	NOR- MAL	S' c	b	S' b	c	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
Female parents hatched at 31.5°C.															
E 3	34	41	21	35	8	14	11	7	171	75	56	22	18	43.2	23.4
9	25	22	24	26	15	12	20	7	151	47	50	27	27	51.1	35.7
13	32	32	34	37	22	24	22	10	213	64	71	46	32	48.2	36.6
G 1	37	44	26	17	14	22	10	10	180	81	43	36	20	35.0	31.1
2	31	32	16	17	12	21	5	4	138	63	33	33	9	30.4	30.4
4	17	30	15	14	20	11	8	11	126	47	29	31	19	38.0	39.6
5	35	45	32	14	14	30	18	7	195	80	46	44	25	36.4	35.4
6	33	47	33	21	14	19	16	7	190	80	54	33	23	40.5	27.8
7	36	44	23	16	11	19	11	18	178	80	39	30	29	38.2	33.1
8	32	35	29	25	29	28	16	16	210	67	54	57	32	40.9	42.3
9	25	35	25	19	24	21	12	12	173	60	44	45	24	39.3	39.9
11	29	46	30	27	27	23	12	11	205	75	57	50	23	39.0	35.6
12	26	46	37	21	21	29	8	13	201	72	58	50	21	39.4	35.3
13	31	40	20	21	12	13	9	5	151	71	41	25	14	36.4	25.8
15	21	22	14	17	10	13	4	2	103	43	31	23	6	35.9	28.1
17	39	36	14	20	17	20	13	4	163	75	34	37	17	31.2	33.1
18	9	12	15	9	9	11	4	7	76	21	24	20	11	46.0	40.5
28	22	21	23	8	8	11	8	11	112	43	31	19	19	44.6	33.9
29	22	19	19	11	13	10	10	5	109	41	30	23	15	41.3	34.8
140	47	27	11	20	19	12	5	8	149	74	31	31	13	29.5	29.5
141	47	46	28	22	22	23	10	9	207	93	50	45	19	33.0	30.8
142	32	45	15	15	11	21	5	3	147	77	30	32	8	25.8	27.2
145	44	55	25	29	29	26	8	5	221	99	54	55	13	30.3	30.7
Total.....									3,769	1,528	990	814	437	37.8	33.2
Second broods of above after 10 days, at 22°C.															
E6 II	29	23	24	24	7	10	4	5	126	52	48	17	9	45.2	20.6
7 II	55	33	27	38	14	17	18	13	215	88	65	31	31	44.5	28.8
9 II	56	46	47	37	25	18	23	13	265	102	84	43	36	45.2	29.8
10 II	53	37	27	33	16	14	12	6	198	90	60	30	18	39.3	24.2
Total.....									804	332	257	121	94	43.6	26.8

TABLE 8

*Complete analysis of effect of temperature on crossingover*

No. 2. Tube counts in table 14

No. 3 and No. 8. Summaries in table 5

No. 4. b pr c

Control summaries in table 11, No. 5.

NUMBER	NOR- MAL	b, pr c	pr, c	b	c	b, pr c	b, pr c	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
Female parents hatched at 17.5°C.															
														per cent	per cent
280	117	109	5	11	30	46	4	0	322	226	16	76	4	6.2	24.8
281	96	75	9	6	21	25	1	0	233	171	15	46	1	6.9	20.2
282	87	88	9	13	24	35	3	4	263	175	22	22	7	11.0	25.1
283	126	100	10	9	19	30	1	4	299	226	19	49	5	8.0	18.1
285	140	135	8	11	39	55	4	2	394	275	19	94	6	6.3	25.4
286	123	105	12	17	39	30	2	3	331	228	29	69	5	10.3	22.3
287	138	88	13	10	26	34	1	2	312	226	23	60	3	8.3	20.2
288	136	147	16	13	45	49	6	5	417	283	19	94	11	9.6	25.2
289	143	68	9	8	26	37	4	4	299	211	17	63	8	8.3	23.7
Total .....									2,870	2,021	189	610	50	8.3	23.0

Control summaries in table 17, No. 2

No. 6.  $\frac{S'}{b \quad c}$ 

NUMBER	S'	b, c	S', b c	NOR- MAL	S' c	b	S' b	c	TOTAL	NON. CROSS- OVER	1	2	1-2	I'	II
Female parents hatched at 27°C.															
														per cent	per cent
52	81	59	41	52	20	29	17	15	314	140	93	49	32	39.8	25.8
53	55	61	36	67	13	19	5	8	264	116	103	32	13	43.9	17.0
54	105	88	68	77	41	36	20	19	454	193	145	77	39	40.5	25.5
55	48	45	23	38	14	14	6	8	196	93	61	28	14	38.2	21.4
57	72	64	29	32	9	9	7	2	224	136	61	18	9	31.2	12.0
58	70	60	32	42	9	18	6	11	248	130	74	27	17	36.3	17.7
59	86	102	59	62	21	20	9	8	367	188	121	41	17	37.6	15.9
60	74	82	41	63	8	22	4	8	302	156	104	30	12	38.4	13.9
61	59	82	40	49	26	19	7	10	292	141	89	45	17	36.3	21.2
62	86	74	50	51	12	19	10	7	309	160	101	31	17	36.1	15.5
63	73	83	43	60	12	17	13	12	313	156	103	29	25	40.9	17.3
64	62	63	43	51	14	22	10	11	276	125	94	36	21	41.6	20.7
Total .....									3,559	1,734	1,149	443	233	38.9	19.0

TABLE 8—Continued

b pr c  
No. 7 and No. 9

NUMBER	NOR- MAL	b, pr c	b	pr, c	b, pr c	c	b, c	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	1	11
Control. Female parents hatched at 22°C.															
514	84	64	4	4	15	20	4	0	195	148	8	35	4	6.1	20.0
515	95	70	4	5	19	15	0	1	210	165	9	34	2	5.2	17.1
516	90	59	6	9	24	19	3	2	212	149	15	43	5	9.4	22.6
517	59	53	4	4	14	12	0	2	148	112	8	26	2	6.3	17.7
518	113	84	7	6	20	23	1	1	255	197	13	43	2	5.9	16.8
519	95	90	5	5	30	19	0	0	254	185	20	49	0	7.8	19.2
520	98	85	8	9	34	24	2	2	262	183	17	58	4	8.0	23.6
521	85	89	5	6	31	18	0	1	235	174	11	49	1	5.1	21.2
522	91	77	2	6	20	27	1	1	225	168	8	47	2	4.4	21.6
523	97	100	9	3	22	15	2	1	249	197	12	37	3	6.0	16.0
524	97	70	8	3	39	22	5	1	245	167	11	61	6	6.9	27.3
525	95	90	5	5	29	21	3	1	249	185	10	50	4	5.6	21.6
526	85	85	2	5	26	14	1	1	219	170	7	40	2	4.1	19.1
527	83	90	1	3	25	27	3	0	232	173	4	52	3	3.0	23.6
528	120	122	7	7	29	29	0	5	319	242	14	58	5	5.9	19.7
561	100	93	7	3	30	19	2	3	267	193	20	49	5	9.3	20.2
562	108	107	7	8	29	24	0	0	283	215	15	53	0	5.3	18.7
563	123	95	9	4	33	30	0	2	296	218	13	63	2	5.1	21.9
564	84	65	3	5	17	23	2	0	199	149	8	40	2	5.0	21.1
565	110	108	5	3	22	18	1	1	268	218	8	40	2	3.7	15.6
Total.....									4,822	3,608	231	927	56	5.9	20.3

No. 7. Female parents hatched at 29°C.

529	98	100	8	14	22	22	4	3	271	198	22	44	7	10.7	18.8
530	105	66	5	7	11	15	1	0	210	171	12	26	1	6.2	12.8
531	96	59	3	5	13	17	3	0	196	155	8	30	3	5.6	16.8
532	99	67	9	7	28	26	1	1	238	166	16	54	2	7.5	23.5
533	106	69	11	7	28	23	1	3	248	175	18	51	4	8.8	22.1
534	89	98	16	19	29	36	1	4	292	187	35	65	5	13.6	23.9
535	78	87	5	6	23	24	2	0	225	165	11	47	2	5.7	21.7
536	109	107	10	15	31	16	4	1	294	216	25	47	6	10.5	18.0
537	72	78	4	9	24	23	3	0	213	150	13	47	3	7.5	23.5
540	75	77	15	4	27	16	2	5	221	152	19	43	7	11.7	22.6
542	90	82	12	6	28	38	1	2	259	172	18	66	3	8.1	26.6
543	89	81	7	10	28	33	2	1	251	170	17	61	3	7.9	25.5



TABLE 8—Continued

NUMBER	NOR- MAL	b, pr c	b	pr, c	b, pr c	c	b, c	pr	TOTAL	NON- CROSS- OVER	1	2	1-2	I	II
														per cent	per cent
566	129	97	10	16	49	33	2	1	337	226	26	82	3	8.6	25.2
567	71	61	11	5	21	23	2	1	195	132	16	44	3	9.7	23.6
568	119	83	10	9	34	33	3	2	293	202	19	67	5	8.2	24.5
569	85	84	8	9	25	26	1	1	239	169	17	51	2	7.9	22.2
570	99	98	15	8	31	32	2	2	287	197	23	63	4	9.4	23.4
Total.....									4,269	2,993	315	898	63	8.8	22.5

No. 9. Female parents hatched at 32°C.

549	68	63	8	10	16	21	4	4	194	131	18	37	8	13.4	23.2
552.2	55	35	5	11	12	12	1	4	135	90	16	24	5	15.5	21.4
552.3	44	36	6	6	11	18	5	2	118	80	12	19	7	16.1	22.0
571	68	49	17	11	24	15	4	4	192	117	28	39	8	18.7	24.5
573	86	87	17	10	33	24	3	6	266	173	27	57	9	13.6	24.8
574	89	83	14	14	32	38	4	6	280	172	28	70	10	13.6	28.6
575	83	56	13	23	36	39	4	11	265	139	36	75	15	19.2	37.7
576	63	70	18	16	27	23	4	7	228	133	34	50	11	19.7	26.8
577	50	30	12	11	16	18	1	2	140	80	23	34	3	18.5	26.3
578	81	74	18	18	29	22	5	3	250	155	36	51	8	17.6	23.5
580	118	113	15	12	38	31	3	6	336	231	27	69	9	17.4	23.2
582	47	63	10	8	24	16	5	2	175	110	18	40	7	14.3	26.9
583	49	53	14	11	18	29	3	3	180	102	25	47	6	17.2	29.3
585	92	104	19	22	33	44	7	6	327	196	41	77	13	13.5	27.5
586	48	29	5	9	16	9	1	3	120	77	14	25	4	15.0	24.0
587	67	75	16	12	26	39	3	4	242	142	28	65	7	14.5	29.8
588	69	65	7	14	29	24	5	7	220	134	21	53	12	13.7	26.9
589	62	52	9	10	15	19	3	4	174	114	19	34	7	15.0	23.6
590	109	96	17	22	51	32	11	8	346	205	39	83	19	16.8	29.5
591	61	59	11	12	20	15	4	6	188	120	23	35	10	17.6	24.0
Total.....									4,376	2,701	513	984	178	15.7	26.5

TABLE 9

*Effect of temperature on crossingover when C II l is present*

S' b C Ill c sp															
NUMBER	S' b	c, sp	S' b c sp	NORMAL	S' b sp	c	S' b c	sp	TOTAL	NON-CROSS-OVER	1	2	1-2	S c	c sp
Control. Female parents hatched at 22°															
24	51	51	20	26	28	25	10	13	223	101	46	53	23	per cent	per cent
25	63	73	25	26	40	49	7	8	291	136	51	89	15		
27	65	68	32	38	46	49	14	14	326	133	70	95	28		
32	47	32	24	31	29	27	9	4	203	79	55	56	13		
33	68	75	30	45	39	47	6	12	322	143	75	86	18		
34	46	45	19	24	34	32	8	5	213	91	43	66	13		
44	65	53	16	22	49	42	7	7	261	118	38	91	14		
45	55	61	16	19	27	31	5	4	218	116	35	58	9		
Total .....									2,057	917	413	594	133	26.6	35.3
Second broods of above															
65 (27)	79	71	25	26	43	45	5	7	301	150	51	88	12		
66 (32)	53	56	19	23	33	26	4	7	221	109	42	59	11		
67 (33)	89	82	35	36	38	41	3	6	330	171	71	79	9		
93 (45)	75	71	23	29	60	51	6	3	318	146	52	111	9		
Total .....									1,170	576	216	337	41	21.9	32.3
Female parents hatched at 31.5° C.															
120	36	46	18	16	27	19	4	7	173	82	34	46	11		
121	36	31	7	14	14	14	4	5	125	67	21	28	9		
123	59	65	23	35	30	42	7	12	273	124	58	72	19		
124	48	48	23	17	24	22	10	7	195	96	40	42	17		
125	52	59	22	14	32	33	7	4	223	111	36	65	11		
126	46	46	14	9	21	21	3	2	162	92	23	42	5		
127	47	52	16	21	25	30	7	4	202	99	37	55	11		
Total .....									1,353	671	249	350	83	24.5	32.0
Second brood after ten days at 22°C.															
101	62	46	18	23	26	28	5	3	211	108	41	54	8	22.7	29.0

TABLE 11

*Effect of temperature on second and third broods*

$$\begin{array}{c} S' \\ \hline b \quad c \end{array}$$

NUMBER	S'	b	c	S', b	NORMAL	S'	b	c	S', b	c	TOTAL	NON-CROSS-OVER	1	2	1-2	I	II
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No. 1. Control. Female parents hatched at 22°C.

															<i>per cent</i>	<i>per cent</i>
147	68	96	31	40	17	27	5	6	290	164	71	44	11	27.8	19.0	
148	72	63	44	52	14	35	10	9	299	135	96	49	19	38.4	22.7	
150	72	82	63	35	16	29	6	7	310	154	98	45	13	35.8	18.7	
151	64	62	51	55	22	24	9	9	296	126	106	46	18	38.0	21.6	
Total.....									1,195	579	371	184	61	36.1	20.5	

No. 2. Second broods after ten days at 22°C.

147A	84	96	24	41	16	18	5	2	286	180	65	34	7	25.2	14.3
148A	79	97	44	60	13	20	3	5	321	176	104	33	8	34.8	12.7
150A	94	113	49	58	22	18	3	7	364	207	107	40	10	32.1	13.7
151A	91	107	43	52	26	28	3	10	360	198	95	54	13	30.0	18.6
Total.....									1,331	761	371	161	38	30.5	14.9

No. 3. Female parents hatched at 31.5°C.

140	47	27	11	20	19	12	5	8	149	74	31	31	13	29.5	29.5
141	47	46	28	22	22	23	10	9	207	93	50	45	19	33.0	30.8
142	32	45	15	15	11	21	5	3	147	77	30	32	8	25.8	27.2
145	44	55	25	29	29	26	8	5	221	99	54	55	13	30.3	30.7
Total.....									724	343	165	163	53	30.1	29.8

No. 4. Second brood after ten days at 22°C.

140A	35	35	29	29	16	14	5	5	168	70	58	30	10	40.5	23.7	
141A	72	92	41	50	15	20	5	7	302	164	91	35	12	34.7	15.6	
145A	55	55	32	21	8	12	6	6	195	110	53	20	12	33.3	16.4	
Total.....										665	344	202	85	34	35.4	17.8

TABLE 11—Continued

b pr c															
NUMBER	NORMAL	b, pr c	b	pr c	b pr	c	b c	pr	TOTAL	NON-CROSS- OVER	1	2	1-2	I	II
No. 5. Control. Female parents hatched at 22°C.															
220	150	84	13	8	32	30	5	5	327	234	21	62	10	9.4	22.0
222	157	126	9	6	31	42	0	1	372	283	15	73	1	4.3	19.8
223	163	93	2	4	16	20	2	3	303	256	6	36	5	3.6	13.2
224	157	116	9	10	27	42	3	0	364	273	19	69	3	5.7	19.7
238	134	136	7	9	37	24	1	2	350	270	16	61	3	5.4	18.2
239	98	104	5	9	29	28	1	0	274	202	14	57	1	5.4	20.7
240	88	72	6	11	26	25	1	0	229	160	17	51	1	7.8	22.7
Total.....									2,219	1,678	108	409	24	5.9	19.5
No. 6. Second brood after ten days at 22°C.															
220I	121	67	1	1	26	10	0	0	226	188	2	36	0	0.8	15.9
222I	117	82	4	2	6	17	0	1	229	199	6	23	1	3.0	10.4
224I	122	101	1	2	32	29	0	0	287	223	3	61	0	1.0	21.2
238I	152	115	1	4	32	19	1	0	324	261	5	51	1	1.8	16.0
Total.....									966	777	16	171	2	1.8	17.9
No. 7. Third broods after twenty days at 22°C.															
220II	66	58	3	3	15	15	1	0	161	124	6	30	1	4.3	19.3
222II	65	71	1	3	10	16	0	0	166	136	4	26	0	2.4	15.7
224II	64	46	2	2	12	9	0	0	135	110	4	21	0	2.9	15.5
226II	66	56	5	4	12	19	1	1	164	122	9	31	2	6.7	20.1
238II	48	31	1	0	8	11	0	1	100	79	1	19	1	2.0	20.0
Total.....									726	571	24	127	4	3.8	18.0
No. 8. Second broods after ten days at 31.5°C.															
226I	56	65	18	8	34	25	7	6	219	121	26	59	13	17.8	32.7
239I	24	24	10	8	10	12	6	3	97	48	18	22	9	27.8	31.9
Total.....									316	169	44	81	22	20.8	32.6
No. 9. Females hatched at 31.5°C.															
470	58	51	11	14	25	29	5	1	194	109	25	54	6	15.9	30.9
473	58	28	8	9	25	16	5	2	151	86	17	41	7	15.8	31.7
Total.....									345	195	42	95	13	15.8	31.3
No. 10. Second brood after ten days at 21.5 C.															
470A	52	27	8	9	6	14	0	2	118	79	17	20	2	16.1	18.6

No. 4. Control. See No. 1, table 11



## THE PHOTIC SENSITIVITY OF BALANOGLOSSUS<sup>1</sup>

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In view of the fact that the Balanoglossida have enjoyed so considerable a morphological prominence, by reason of the affinities to the early vertebrate stock attributed to them, it is rather a matter for surprise that information regarding their behavior and activities should be, as it is, conspicuously lacking. Owing to the somewhat unusual condition of the nervous system in these animals, moreover, their responses should, when analysed, provide some data of general interest. Perhaps the absence of data upon the behavior of balanoglossids is in part due to the difficulty of obtaining them entire and in quantities ample for purposes of experimentation. Certain of the more highly modified balanoglossids (*Ptychodera*, *Glossobalanus*) are available at Bermuda, and after devoting some time to a study of their local distribution, I have been able to procure several species in quantities sufficient for study.

One of these forms, probably *Ptychodera bahamensis* Spengel, has proved to be very plentiful, and the greater part of the observations here described were made upon individuals of this species. The present paper aims merely to record certain responses of *Ptychodera* when exposed to stimulation by light.

These animals occur in localities which are at some distance from the laboratory of the Biological Station, and when brought to the laboratory for observation they apparently suffer somewhat from the disturbances incident to transportation. This is particularly true in the warmer summer months. When kept in aquaria, either with or without sand, they, like other enteropneusts (cf. Caullery et Mesnil, '04), exhibit hardly any active movements, unless they be violently disturbed.- By careful

<sup>1</sup>Contributions from the Bermuda Biological Station for Research. No. 72.

observation, however, certain characteristic types of reaction may be made out.

If the animals are handled with care during collection and subsequently, they may be kept in a healthy condition for some time. Certain preliminary trials with specimens obtained in the summer time led me to state (Crozier '15) that *Ptychodera* was sensitive to sudden changes of light intensity, but that it was not phototropic.<sup>2</sup> Subsequent work with animals in better condition, and particularly with numerous individuals collected during the cooler winter period, has shown this statement to be incomplete. *Ptychodera* is, in fact, definitely phototropic when the animal is in physiologically good condition.

No observations seem previously to have been made with reference to the sensitivity of *Balanoglossus* toward light. Considering their habits in natural circumstances, one would be led to suppose that these enteropneusts would tend to move away from a source of light, or that they would retract as the result of suddenly increased illumination. Assheton ('08) states that the *Dolichoglossus* studied by him protruded its long proboscis from the mud only at night. The *Ptychoderas* studied by me are found in sands of rather well defined types, either just below the surface of the sand or between this surface and the under side of flat stones. When uncovered, they promptly burrow again, or, if merely the proboscis and collar have been exposed, draw back into the sand.

A series of tests was made in which the animals were placed in an oblong aquarium jar and illuminated from one side. The vessel was in a dark-chamber. In the first trials the aquarium contained clean sea-water without any sand. The animals were selected from freshly collected stock living in large glass dishes provided with a good supply of their native sand. I soon found that the *Ptychoderas* could move about much better if some sand was also placed in the experimental aquarium.

After being in the dark-chamber for some time before light was admitted, sunlight reflected from a mirror was allowed to

<sup>2</sup> In the hands of the ingenious type setter, this word has, in the note cited, become transformed into 'photographic.'



fall upon the Ptychoderas. They immediately gave evidence of increased peristaltic activity, and those individuals judged to be in good condition soon turned the proboscis away from the light, and began to creep in the same direction. When illuminated in this way the proboscis becomes very mobile, and peristaltic waves pass rapidly along the genital pleurae, thorax and abdomen. The collar also exhibited wavy motions, particularly along its anterior edge.

When Ptychodera, or Glossobalanus, is illuminated from the side, it promptly turns the proboscis end toward the opposite side. Neither here nor in its movements after orientation is accomplished does Ptychodera make any 'mistakes'; its movements are directed immediately away from the light.

Fragmented animals were also employed, and it was found that anterior halves, separated in the region of the genito-hepatic transition, oriented in a normal manner and moved away from the light. Posterior halves, and pieces of the abdomen (including the caudal extremity) were stimulated to slightly increased peristaltic movement, and progressed away from the light tail first. Isolated posterior fragments tend to move in a caudad direction, even in the dark, but otherwise show relatively little in the way of organized activity until they have begun to regenerate.

When complete Ptychoderas, or anterior fragments, are placed ventral side uppermost they continue for a time their usual peristaltic movements, but progress in a caudad direction. Animals so situated, when exposed to light thrown on the anterior end, contract lengthwise to a notable extent, but continue to move backward. The reversed direction of locomotion, which results from the abnormal position of the balanoglossid, is a consequence of its normal peristaltic motions, and of the fact that it cannot, when so situated, adhere to the substratum at the anterior end.

It may be mentioned that a Ptychodera from which the collar nervous system (the delaminated part) has been removed, but otherwise intact, will orient in the usual way. These specimens, as well as the normal individuals, were found to burrow with the proboscis turned away from the light.

Examination of the surface of many individuals of *Ptychodera* with a small intense beam of light (cf. Patten, '15) showed that the only part which is conspicuously sensitive is the very tip of the proboscis. In the *Tornaria* larva there are found, in the apical region, which becomes the tip of the proboscis, two well defined groups of sensory cells, which have been termed eyes (cf. Bourne, '89; Morgan, '94; etc.). Ritter and Davis ('04, p. 195) state, however, that the *Tornaria* examined by them is not reactive to light. Certain annelid larvae are, as is well known, markedly phototactic, and possess organs corresponding to the 'eyes' of *Tornaria* (cf. Gerould, '06, p. 105). The behavior of *Tornaria* might, therefore, bear reexamination on this point.

The 'eyes' of the larva disappear, however, as the adult form is gradually assumed. No trace of them has yet been located in the proboscis of later post-larval stages. Nevertheless this region of the animal remains markedly sensitive to light. The whole tip of the proboscis, nearly one-fifth its length, seems about equally sensitive and I have been quite unable to establish any bilateral distribution of sensitive parts. The course of phototropic orientation in *Ptychodera* agrees with this condition, for the proboscis is slowly pushed from side to side as the animal moves away from the light. The extent of the sideward excursions is never considerable, unless the incident light be strictly horizontal, in which case the creature's proboscis may largely be shaded by its own body. When the light is made to fall at a slight angle, so that this shading is avoided, orientation is much more precise. *Ptychodera* illustrates the principle that an animal having an axial photosensitive spot may yet by suitable movements convert this terminal area into a bilateral sense organ.

Photic sensitivity in *Ptychodera* may easily be separated from that to tactile or chemical excitation. If the animals be strongly illuminated for some time, they cease to respond to light after a brief intermission in darkness. Cocaine hydrochloride or chlorotone, added to the water, likewise abolish photic irritability before tactile and chemical responses are notably

interfered with. This is, of itself, insufficient proof of the separateness of the sensory organs concerned in the reception of photic stimulation, but it does show that photic irritability depends upon a process distinct from that implicated in the other modes of stimulation.

The general surface of the balanoglossids which I have employed is also sensitive to light, as previously stated, although this sensitivity is much lower than that of the proboscis-tip. Local movements begin rather promptly when bright light is thrown upon portions of the animal; and even when an animal is photically so exhausted that orientation no longer results, there is still some increase in movement when the luminous intensity is suddenly increased.

Light has also another effect on *Ptychodera*, and on *Glossobalanus*. It is known that sunlight inhibits the production of light by certain animals. One such case was carefully studied by Peters ('05), who showed that the phosphorescence of the ctenophore *Mnemiopsis* was inhibited by sunlight, although its recovery of luminous powers in the dark was accelerated if the animal was simultaneously agitated by mechanical means.

All the Enteropneusta with which I am acquainted can produce a vivid greenish light, from every portion of the body save the gills. When placed in a dark room during daylight hours, they do not exhibit their phosphorescence in response to mechanical stimulations of various kinds. And even at night, after they have been in darkness for some time, five minutes' illumination by the relatively feeble light from a twenty-five watt tungsten filament, placed fifteen feet from the aquarium, was found to make it more difficult to elicit the phosphorescent response. When a number of individuals were placed in the early morning in a dark-room, it was found impossible six hours later to obtain light production in response to mechanical stimulation unless the animals were strongly pressed or struck. Induction shocks of moderate strength, however, did induce light production at any time, whether the *Ptychodera* had been in darkness or in light.

It is clear, then, that light exercises a distinctly inhibitory effect on light production by these enteropneusta. Small fragments of the balanoglossids will, if kept in darkness, respond at night to a blow struck on the side of their container, by the emission of a bright glow. But even these isolated pieces are affected by bright light just as are the complete animals. Hence a central nervous effect is eliminated, and it is legitimate to state that light can act directly on *Ptychodera* (and *Glossobalanus*) in such a way as to inhibit the excretion of light-producing substances. I was unable to carry out an experiment which would submit one portion of a *Ptychodera* to light while the rest remained in darkness, and can therefore say nothing about the possible nervous transmission of this kind of photic influence.

#### SUMMARY

Balanoglossids are here described which are photokinetic, and orient away from the light in a machine-like manner. In addition to the orienting stimulus, it is shown that light has another, possibly separate, effect upon these animals, namely the inhibition of light production. The tip of the proboscis is the part most sensitive to illumination, but the rest of the animal's surface is likewise open to stimulation by light. The collar nervous system (delaminated part) is unnecessary for the coordinated movements of orientation, and also for the inhibitory influence of light on the production of luminescence.

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## THE POWER OF SUCTION IN THE SEA-ANEMONE CRIBRINA<sup>1</sup>

G. H. PARKER

ONE FIGURE

*Cribrina xanthogrammica* Brandt, the common sea-anemone of the west coast of North America, is remarkable for its powers of suction. If a piece of smooth wood is moved about among the tentacles of an expanded *Cribrina*, the outer ends of these organs fasten temporarily to the wood by suction and hold to it with no small force. If the finger of the experimenter is used instead of the wood, the tentacles seize it and by contraction attempt to pull it toward the animal's mouth. The feeling of suction where the tentacle is in contact with the finger is considerable. The tentacles attach themselves not only by their tips but also by their sides, especially near their distal ends. Suction at the end of the tentacle seems to be accomplished by a vigorous inversion of the tip, thus forming a pore-like depression. On the sides suction is brought about by the formation of a short vertical groove.

Tentacular suction, as might be expected, is concerned with feeding. This is well illustrated by a single example. A small fish about 4 cm. long was liberated in a glass aquarium in which a *Cribrina* had settled. By accident the fish soon brushed across the tentacles of the sea-anemone, whereupon it was seized and held firmly a few seconds by the sucking ends of the tentacles. Through its struggles it succeeded in escaping, but only to be recaptured soon after. Again it escaped, but in a short time was finally caught. It now seemed much weakened and in part paralyzed. Its skin was whitened in many places and shredded. Doubtless it had felt the effects of the actinian's nematocysts.

<sup>1</sup> Contributions from the Zoological Laboratory of the Museum of comparative Zoology at Harvard College, No. 299. This work was done at the Scripps Institution for Biological Research, La Jolla, California, to the staff of which I wish to express my thanks for many courtesies shown me.

Unable to escape, it was now gradually swallowed by the sea-anemone. Thus the suction exerted by the tentacles is an effective element in enabling the *Cribrina* to catch and hold its prey.

But the tentacles are not the only organs of suction possessed by *Cribrina*. Below the row of whitish acrorhagi that border the oral margin of the column of this sea-anemone run vertical rows of tubercles. Each row extends more or less regularly from the oral to the pedal disc and may contain as many as fifty members. The tubercles are low and terminate externally in a disc-shaped face in the center of which there is a depressed slit or irregular opening. Each tubercle resembles more or less closely the contracted ambulacral foot of a starfish. Most of

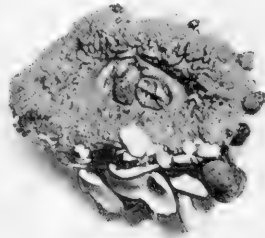


Fig. 1 Oral view of a preserved *Cribrina* showing fragments of shell and pebbles attached to the column; three-fourths natural size. Photograph by Dr. S. Hecht.

the tubercles are well drawn back into the flesh of the sea-anemone but not a few are somewhat extended and have attached to their outer ends fragments of shell or stone (fig. 1). In some instances several tubercles are attached to the same piece of shell, but in many cases each fragment is held by a single tubercle. These bits of shell and stone are so abundant on the column of *Cribrina* that when it contracts they form a complete covering for it and render it almost indistinguishable from the surrounding beach material. This shelly covering probably shields the animal from the force of the waves and from desiccation. It does not seem to be in the nature of a protective resemblance, for apparently these sea-anemones are not eaten by other animals.

When an attempt is made to pull off the fragments of shell



attached to a *Cribrina*, they are found to be securely held in place. Yet if the animal is transferred to a laboratory tank and especially to a vessel of standing water, many of these fragments soon drop off. Gee ('13, p. 307) has observed that the injection of a solution of potassium chloride or of beef juice into a fresh *Cribrina* in a tide pool causes many of the fragments to be shed at once. A denuded *Cribrina* put into a rocky crevice soon covers itself again with fragments of shell and gravel. Apparently the tubercles act as suction organs and quickly take hold of fragments of shell or drop them in accordance with circumstances.

If a *Cribrina* is placed on its side in an aquarium, it soon attaches itself by the suction of those tubercles that are next the bottom of the aquarium and near the pedal disc. It then gradually turns itself on to its pedal disc and eventually the attached tubercles drop their hold. Thus the suction of the tubercles is an essential element in the righting reaction of this sea-anemone.

As it is possible to find fragments of shell of considerable size attached to the sea-anemone by only a single tubercle, it is not difficult to arrange apparatus to measure the force necessary to break the attachment, for when the shell is drawn away from the sea-anemone, it and the tubercle always separate on their natural surfaces.

To accomplish such measurements, a spring balance was adjusted overhead so that a string could be brought from it vertically downward into an open glass vessel in which was a *Cribrina*. The string was now tied to an appropriate fragment of shell and the vessel lowered till the shell and tubercle separated. The force just necessary to accomplish this separation was indicated on the scale of the spring balance and recorded. The area of the attachment of the tubercle to the shell was usually clearly marked on the shell and this was also measured and recorded. Such measurements were facilitated by choosing tubercles that had become attached to the flat face of a bit of shell and whose area of adhesion was bounded by an approximate circle, a not unusual form of attachment. The records from ten trials are given in table 1.

TABLE 1

*Breaking forces (grams), diameters of suction areas (millimeters), calculated areas of suction (square millimeters) and calculated suction-pressure (grams per sq. millimeter) for ten trials in separating fragments of shell from the suction tubercles of Cribrina.*

Breaking forces in grams.....	47	28	29	50	53	38	52	50	65	60	47.2	Averages
Diameters of suction areas in millimeters...	2.6	1.6	1.5	2.6	2.5	1.8	2.9	2.6	3.0	2.9	2.40	
Calculated areas of suction in sq. millimeters.....	5.3	2.0	1.8	5.3	4.9	2.5	6.6	5.3	7.1	6.6	4.74	
Calculated suction-pressure in grams per sq. millimeters...	8.6	13.9	16.5	9.4	10.8	15.0	7.9	9.4	9.2	9.1	11.0	

It will be noted from the table that large breaking forces are usually associated with large suction areas, small ones with small areas. The average breaking force for the ten trials is 47.2 grams and the average suction-pressure 11 grams per square millimeter. This amounts approximately to 15.6 pounds per square inch. As the limit of suction under ordinary circumstances is one atmosphere or 14.7 pounds per square inch, it appears that at the moment of breaking the sea-anemone was exerting probably as much suction as, under the circumstances, was physically possible.

Although the mechanism of the suction apparatus in *Cribrina* has not been worked out, it is without much doubt dependent upon muscle, and muscle is said (Howell, '13, p. 41) to vary in the pull it can exert from 7 (up to 30) grams per square millimeter, in the frog, to 62.4 grams per square millimeter, in man. Hence it may be concluded that, though the suction apparatus in *Cribrina* has reached a physical limit, it has not necessary reached an organic limit.

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## NOTES ON THE LOCOMOTION OF CERTAIN BERMUDIAN MOLLUSKS<sup>1</sup>

J. M. D. OLMSTED

ONE FIGURE

Vlès ('07) has classified the types of gastropod locomotion according to the region of origin and method of progression of the pedal waves. He applies the term direct to those waves which pass over the foot in the direction in which the animal itself is moving, i.e., from posterior to anterior, and retrograde to those which proceed in the opposite direction. He has made a further subdivision with reference to the lateral extent of waves, calling the locomotion monotaxic when each wave extends across the whole width of the foot, di- and tetrataxic when two and four parallel systems, respectively, are exhibited on a foot. Parker ('11) found that it was possible to distinguish two subtypes in ditaxic locomotion, one in which the waves of the two sides alternated in position, and the other in which they were opposite to each other. Parker also made a further addition to the types of gastropod locomotion when he described the "arhythmic pedal movements" of *Illyanassa obsoleta* Say.

In the present paper further examples of several of these types are reported, as well as several new types, which present decided peculiarities. A method of demonstrating that pedal waves are concavities is given, and backward locomotion is described. The work was done at the Bermuda Biological Station in the summer of 1916. I am indebted to Dr. G. H. Parker for suggesting a study of gastropod locomotion, to Dr. W. J. Crozier for his courtesy in turning over material to me, and to Dr. E. L. Mark and to the Trustees of the Humboldt Fund for having made it possible for me to go to Bermuda. My thanks are

<sup>1</sup> Contributions from the Bermuda Biological Station for Research. No. 73.

also due to Mr. W. F. Clapp for the identification of the majority of the mollusks referred to in this paper.

1. The huge slug, *Veronicella schivelyoe* Pilsb., which, according to Verrill ('02), is not known to occur elsewhere than in Bermuda, shows direct monotaxic locomotion, i.e., the pedal waves pass from posterior to anterior and extend the full width of the foot. In *Veronicella*, when the animal is moving, there are always about eleven such waves, a condition which is in strong contrast to another pulmonate abundant in Bermuda, *Onchidium floridanum*, which shows the same type of locomotion, but shows only one or two waves at a given time. The number is fairly constant for individuals of *Veronicella* having very different lengths. It may even be that the number is constant for the species and that the difficulty of counting the rapidly moving waves in the small animals is responsible for the small differences obtained.

LENGTH OF FOOT	AVERAGE NUMBER OF WAVES
<i>cm.</i>	
14.8	11.8
9.6	11.2
9.4	11.3
8.6	11.3
7.9	10.0
3.8	10.5

These animals give an interesting reaction if they are disturbed after having once come to rest. When one taps the animal lightly, as for instance with a pencil, at any point on the posterior third of its dorsal surface, waves appear on the foot varying in number from the whole eleven in the case of a light tap, to five after a strong tap. In the former case the slug usually continues producing pedal waves and moves away, while in the latter, the most posterior waves are the most pronounced, and as they pass anteriorly they diminish in intensity, and all disappear after two or three new waves have been added to the original number. If, however, one taps the slug's head, this part is pulled back and no waves at all appear on the foot. When the animal is moving,

tapping on its posterior third has no effect, unless perhaps to make it move faster, while to tap the head causes the antennae to be drawn in and the waves in the anterior half of the foot to disappear at once. Künkel ('03) found a somewhat similar reaction to be given by several species of slugs of the genus *Limax*. If an actively creeping *Limax* is touched on the back or on the side of the foot, the pedal waves cease for a moment, after which the slug resumes its movement. Species of the slug *Arion*, on the contrary, when stimulated in the same way, cease wave movement and remain contracted for some time.

2. *Eulota similaris* Fer., a snail found in exactly the same habitat as *Veronicella*, has the same type of locomotion. I was able to count the number of waves on the foot in two specimens only.

LENGTH OF FOOT	AVERAGE NUMBER OF WAVES
<i>cm.</i>	
2.7	9
2.1	10

*Eulota* also shows similar reactions to *Veronicella*, for if a moving *Eulota* is pushed forward by a thrust on the posterior of the shell, locomotion proceeds normally and the tentacles are not drawn in; but if the animal is pushed backward by a thrust on the anterior of the shell, the pedal waves cease and the anterior end of the foot loosens its hold on the substrate.

3. *Helcinia convexa* Pfr., 4. *Tethys dactylomela* Rang. (cf. Jordan, '01), and 5. *Fissurella nodosa* Born show retrograde monotaxic locomotion.

6. *Tectarius misricatus* L. exhibits retrograde alternate ditaxic locomotion.

7. *Tritonidea tineta* var. *bermudensis* Dall affords an example of retrograde tetrataxic locomotion. The foot of this snail is a much smaller organ in proportion to the rest of the body than that of other gastropods I have examined. Its average length is 8 mm. and its width 2 mm. Running longitudinally down the center of the foot is a fine white line hardly visible unless the

animal is moving. During locomotion, a wave starts next this middle line, and is followed by another near the outer edge of the foot. Then a wave appears on the other side of the median line, and in turn is followed by a wave near the outer edge of that side. If one considers the foot as divided into quarters by lines running parallel to the long axis of the foot, and if he numbers these quarters from left to right 1, 2, 3, 4, then the waves appear in this order, 2, 1, 3, 4 or 3, 4, 2, 1.

8. *Columbella mercatoria* L. is also retrograde tetrataxic in its locomotion.

9. *Cypraea exanthema* L. exhibits a type of locomotion different from any described by Vlès ('07) or Parker ('11). Only one specimen was brought to the laboratory during the season and the following description is taken from this animal. During the first three or four days in the laboratory it moved constantly in its jar, coming to rest always on a lateral wall. Later, locomotion occurred only when it was disturbed.

The foot was somewhat pointed at the anterior end being more rounded at the posterior. The lateral edges were practically parallel. In repose the length of the foot was 8.5 cm. and the width at the anterior end 6, but when moving the foot was lengthened to about 9.5 and narrowed to 4 cm. At the extreme anterior edge of the foot (fig. 1) was a narrow (0.5 mm.) band of light, almost white tissue, which exhibited a constant rippling motion. This seemed to bear no relation to the ordinary waves appearing on other portions of the foot.<sup>2</sup> I made many attempts to cause the animal to attach itself to a glass plate by some region of its foot other than the extreme anterior part, but in no case was I successful. For the act of attaching, it seemed necessary that the anterior part of the foot, including the white band, be in contact with the substrate, but when the whole foot once became attached I could, by a quick shove, push the anterior three-fourths off the plate, and still the animal was able to cling perfectly with the remaining fourth of its foot. When in this position, it would bend the anterior edge of the foot around and attach it to the other side of the plate, so that its foot was

<sup>2</sup> Cf. Parker's ('11) description of the locomotion of *Illyanassa obsoleta*.

folded over and attached to both surfaces of the glass at once. The animal would then move forward keeping the posterior part of the foot still in contact with the other side of the plate, until gradually the whole foot was on the other side. *Cypraea* moved with greater velocity under these circumstances than at any other time. The average rate was 1 cm. in four seconds. It was noted that the anterior 2 cm. of the foot seemed fairly

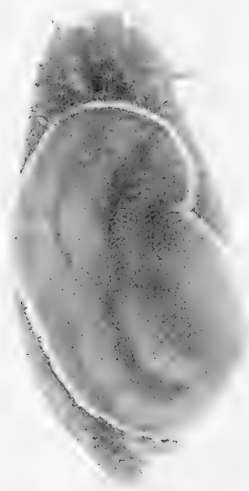


Fig. 1. *Cypraea exanthemia* L. viewed from beneath. The animal is turning to its left. Four anterior lateral waves (dark curved areas) are moving to the animal's left, and three posterior lateral waves to its right.

free from mucus, while a large amount was secreted by the rest of the foot. It may be that the anterior edge of the foot is thus specially modified for the act of attaching the animal to the substrate by suction, and perhaps also for the reception of the stimulus which leads to the attaching reflex. This behavior is in contrast to that of *Chiton* and other mollusks, where any portion of the foot will become attached to a surface (Parker, '14).

But the most striking phenomenon about *Cypraea's* locomotion was the diversity of directions in which the pedal waves were

able to proceed. As a rule the waves moved laterally, i.e., from side to side, instead of from anterior to posterior or the reverse. It was very strange to observe the whole animal moving forward while the waves which caused this movement were actually going sidewise. A single wave presented the appearance of a deep brown streak about 4 mm. wide, bounded anteriorly by a narrow (0.5 mm.) band lighter in color. When the animal was moving forward, waves made their appearance at the posterior edge of the foot, starting like ordinary direct monotaxic waves. But after each one had moved forward 0.5 to 1 cm., it suddenly bent forward, lengthened, and formed a wave which extended the full length of the foot at about 1.5 cm. from either the right or left side. If the wave extended along the left side of the foot, it then travelled to the right, keeping its front parallel to the long axis of the body with the narrow lighter band in advance. In the majority of cases the waves went from left to right, though in several instances the reverse was true. I have called such wave motion lateral, though the resulting locomotion is forward.

One could readily observe the movement of a single point on the foot, since the fine lines present could be used as landmarks. It was seen that as a wave passed over any particular point, it was carried forward, though the wave itself was moving from left to right, or right to left. Parker's ('11) scheme for gastropod locomotion can be applied in this case. The pedal wave is considered as the result of two different muscular contractions; first, contractions of "the dorsoventral muscles lift the foot locally from the substrate," and, secondly, "the contraction of the longitudinal muscles" causes "forward movement of that portion of the foot which is temporarily lifted from the substrate" and "extends the relaxing posterior fibers." But instead of these contractions taking place "in sequence from behind forward," as in *Chiton tuberculatus*—which exhibits retrograde locomotion—in *Cypraea* during lateral wave motion they must occur practically simultaneously along the whole length of the foot, the series of fibers next to contract being those at one side of the contracted ones, instead of those anterior to them. This



produces the same effect as if there were single longitudinal fibers extending the whole length of the foot, each of which contracts in turn.

A second method of forward locomotion was observed in *Cypraea*. Waves often started from the border of the foot between the most posterior point and a point on one side some three centimeters anterior to it, and passed across the foot preserving this same alignment. This therefore made waves which, as they progressed, extended diagonally across the foot, disappearing first anteriorly. In one case while the animal was going forward, such diagonal waves which had been moving from left to right suddenly stopped, and diagonal waves started up in the opposite direction, i.e., from right to left, but without causing cessation of locomotion or a change in the direction of the animal's movement. When one watched a particular spot on the foot over which diagonal waves were passing, it was evident that the spot was carried both forward and sidewise. In this case one must suppose that another set of muscles running transversely in the foot is also in operation, and that both longitudinal and transverse muscles act simultaneously, producing locomotion in a direction demanded by the law of parallelogram of forces. It would seem therefore that the whole animal, instead of moving straight forward, was probably moving forward and to one side at the same time. The distances traveled by the animal were so short (1 to 2 cm. at a time) that I was unable to determine this accurately. If this supposition is true, a periodic reversal of direction of diagonal waves would keep the animal in an essentially straight path.

Forward locomotion, however, was seldom observed in *Cypraea*. Usually the animal kept turning and moving in circles, now to the right, now to the left; if to the left, waves started in the anterior third of the foot about one-half centimeter in from the right edge, and extended from the extreme anterior end backward about one-third the length of the foot. These (lateral) waves passed sidewise from right to left. At the same time lateral waves started on the left side, extending from the center of that side to the posterior end. Not only could there be two sets of

waves moving in opposite directions, but in several cases three separate sets were to be seen. Once, when the animal was turning to the left, waves in the anterior third passed from right to left, in the middle third a second set passed from right to left, but at a different rate and not continuous with the anterior waves, while in the posterior third of the foot, waves were moving from left to right. At another time, when the animal was turning to the right, waves in the anterior part of the foot passed from left to right, in the middle from right to left, and in the posterior from right to left. In every instance, however, the waves in the anterior third were moving in the same direction that the whole animal moved, and in the majority of instances the waves in the posterior third passed in the opposite direction (fig. 1). This would naturally accomplish a quicker turn than any other combination, and would therefore be what one might expect to find. Several instances were, however, noted in which the posterior waves were moving in the same direction as the anterior ones.

A still further complication occurred when an antero-posterior wave appeared at the same time with the lateral waves. This retrograde wave was seen only when the animal was turning rapidly. It occurred on the inside of the curve which the foot made when bending around, i.e., if the animal was turning to the left, the retrograde wave appeared on that, the shortened, side. It was a much wider wave than any of the others, had no light area preceding the brown band, and extended as a rule only half the width of the foot. It did not seem to interfere with the lateral waves, which moved perpendicularly to it. The two waves passed over each other and neither was interrupted. One could see the lateral wave anterior and posterior to the retrograde wave, and the retrograde wave on each side of the lateral one. To this type of locomotion I have given the name composite.

Another strange condition was observed when the *Cypraea* was held at such an angle with a glass plate that it could attach only the very anterior end of the foot. Waves appeared spreading out in a fan-shaped manner from the center of the foot, in some cases half of them going to the right and half to the left,

in other cases, all to the right or all to the left. The foot attached progressively more and more of its surface to the glass, and when two-thirds were adhering, it became evident that these waves were the ordinary lateral waves of the anterior third of the foot, and that they presented this appearance because of the contraction of the unattached portion of the foot. The interesting point was that even in a given portion of the foot waves could go both to the right and to the left at the same time.

When one pushed backward with gradually increasing force on the shell of *Cypraea*, the posterior third of the foot became the same light color as the narrow band which preceded the dark portion of the lateral wave, and at the same time lateral waves started in opposite directions in the anterior and middle thirds. Similar results were obtained when one pushed forward or to the right or left on the shell, a lighter colored area appearing on the side opposite to that on which the force was being applied, waves likewise being set up in the rest of the foot. Like many other mollusks, it was very difficult to dislodge *Cypraea* by a steady push, but this was easily accomplished by a sudden thrust.

The different forms of waves which may therefore appear on the foot of *Cypraea* are (1) long lateral waves, which extend the whole length of the foot and move either to the right or to the left; (2) short lateral waves, which are about one-third the length of the first, two or three sets appearing at one time, the waves in any set moving either to the right or left or half of them to the right and half to the left; (3) diagonal waves, which extend the whole distance across the foot and move either to the right or the left; (4) retrograde waves, which extend from the anterior edge of the foot to the center and, of course, run from anterior to posterior.

10. *Marginella arena* Val. moves much faster than any other marine mollusk that I examined. The average length of the foot is only 1.2 cm. and it moves at the average rate of 1 cm. in 2.4 seconds. No waves at all were discernable on the foot of this gastropod. The animal is so small and so slippery with mucus, that one cannot hold it in the fingers to test the foot with carmine for ciliary action. I therefore attached the shell to a

glass rod by means of "orange sticky wax" and was thus enabled to observe the effect of placing powdered carmine on the foot. The grains of carmine were caught in the mucus and rapidly conveyed posteriorly. I then teased bits of the foot and found upon microscopical examination that long and vigorously beating cilia were present all over the surface of the foot. Those along the edges seemed to beat the more vigorously. This, so far as I know, is the first example of locomotion by ciliary action alone reported for a marine gastropod.

11. The foot of *Haminea antillarum* Orb. also exhibited no waves. This gastropod secretes a very large amount of mucus. If one draws a glass rod along the bottom of the dish across the animal's track, a centimeter or less behind the animal, it is possible to swing the animals around by means of the tough mucus thread which it has left behind itself. Carmine grains sprinkled on the foot are carried posteriorly. Microscopic examination of the teased foot showed cilia on all parts.

12. *Bulla occidentalis* A. Ads. also has a ciliated foot and shows no pedal waves. *Bulla* and *Haminea* move very much more slowly than *Marginella*. Only one specimen of *Bulla* was found. The length of the foot was 1.3 cm. and the animal moved on an average 1 cm. in 20.9 seconds. *Haminea*'s rate is similar.

In enumerating the types of gastropod locomotion, one should not omit the peculiar swimming motion of the 'sea-hares,' such as *Aplysia limacina* and *Tethys dactyliomela*. This is accomplished by wing-like appendages of the mantle, the parapodia. The two gastropods named can also move over a surface by means of retrograde pedal waves.

The following outline gives the relations of all the types of gastropod locomotion which have been described, with the exception of the gallop of *Helix dupetithoutarsi* (Carlson, '05, Jordan, '05; Parker '11).

- I. Parapodial.
- II. Pedal.
  - A. Arrhythmic.
    - 1. Ciliary.
    - 2. Muscular.
  - B. Rhythmic.
    - 1. Direct.
      - a. Monotaxic. b. Ditaxic.  $\begin{cases} (1) \text{ Alternate.} \\ (2) \text{ Opposite.} \end{cases}$  c. Tetrataxic.
    - 2. Retrograde.
      - a. Monotaxic. b. Ditaxic. c. Tetrataxic.
    - 3. Diagonal.
      - a. Monotaxic. b. Ditaxic.
    - 4. Lateral.
      - a. Monotaxic. b. Ditaxic. c. Tritaxic.
    - 5. Composite.

Parker ('14) has briefly described backward locomotion in *Chiton tuberculatus*. I was able to study the animal's backward locomotion in detail by allowing a *Chiton* to attach itself by the posterior portion of the foot to the lower edge of a glass plate held vertically in the air. The following record is typical.

*Experiment Four.* *Chiton C.* Length of foot 7 cm. Attached by 1.5 cm. of posterior end of foot to plate. Waves began at once.

- 1st wave carries foot 0.6 cm. up plate.
- 2d wave carries foot 1 cm. up plate.
- 3d wave carries foot 1.1 cm. up plate.
- 4th wave carries foot 1.1 cm. up plate.
- 5th wave carries foot 0.1 cm. up plate.
- 6th wave. Body turns and foot moves forward 0.3 cm.
- 7th wave. Head against plate. Rim of shell still extending beyond plate.
- Further turning. No forward progression.
- 8th wave. Whole animal against plate.
- 9th to 13th waves carry animal forward.

In this trial the *Chiton* moved straight backward a distance of 3.9 cm. in a series of five waves before it began to turn. In many specimens turning began earlier, e.g. with the second or third wave. One-fourth of the foot was the least that would become attached and remain long enough for waves to carry the whole foot up on to the plate. After a number of trials with the same individual, the animal would finally back and turn just

enough to place the whole foot in contact with the plate. This is a similar result to that which Parker ('11) found with an exhausted *Helix pomatia*.

The ordinary pedal waves which carry Chiton forward are composed of two bands, the first lighter in color than the rest of the foot and often 1.5 cm. broad, the second following the first and darker in color, usually half a centimeter in breadth. The wave which carries Chiton backward moves in exactly the same direction as the other, i.e., is retrograde, but has no lighter portion. It consists solely of the very dark band and is about 0.7 cm. broad. It can be seen that this area is one of great contraction since it may be raised from the substrate 2 mm. or more.

For backward movement Parker's ('11) scheme holds good, the only change being that the fixed point of each longitudinal fiber during contraction is the posterior instead of the anterior end. The wave would then travel in the same direction as before, but the animal would proceed in the opposite direction.

Experiments were also undertaken to determine whether Chiton would 'back' when attached to the top and sides of a vertical plate by the posterior end of the foot. The weight of the body seemed to influence the reactions in both cases. When attached to the side, the first wave only would carry the foot in a straight horizontal direction, all the succeeding waves carried the animal diagonally, a combination of backward and turning movements. When attached to the upper edge of the plate, the Chiton would bend over and attach the anterior end of the foot to the other side of the plate if less than one-fourth of the foot was attached. But if one-fourth or more of the foot is attached, the animal will, without turning, back on to the plate, covering a distance of 1.5 cm. in 4 waves. Twelve trials were made where the individual was balanced across the upper edge of the plate and allowed to attach as it would. Five times it attached the anterior end of the foot and moved forward, seven times the posterior end was attached and the animal backed down the glass. In nearly every trial the Chiton would raise its anterior end and thus upset the balance, causing the posterior end of the foot to touch the plate. Nevertheless in nearly half

the trials contact with the plate did not cause the posterior end to become attached.

The only other mollusk I was able to cause to move backward was the keyhole limpet *Fissurella*. When the posterior fourth or more of its foot was attached to the lower edge of a vertical glass plate, the head of the animal being directed downward, the first wave or two would carry the limpet straight backward for a distance of 2 millimeters or more, then turning would take place. Here again the direction of the wave which carried the animal backward was the same as that which carried it forward. All other mollusks tried were so flexible or had such a large foot that they would bend over and attach the anterior part of the foot to the other side of the glass. Naturally conditions under which these experiments were conducted would hardly be realized in nature. Backward locomotion is probably seldom resorted to by gastropods, at least for any considerable distance, since turning took place sooner or later in every trial. Nevertheless certain mollusks possess the ability to perform such movements.

A clear demonstration that pedal waves are concavities and not convexities (Biedermann, '05; von Uexküll, '09; Parker, '11) is afforded by the use of a manometer. A hole 1 mm. in diameter was bored in a glass plate. Under this was fastened by an air-tight joint of "orange sticky wax" one end of a capillary tube which was bent back on itself in two places forming a letter 's'. The tube was marked off in millimeters and a colored solution was introduced so that any change in level could be readily detected. This capillary manometer was used by placing a mollusk on the underside of the plate and watching the liquid as the animal passed over the hole. For every gastropod tried—*Cypraea*, *Veronicella*, *Onchidium*, *Tectarius*, *Fissurella*, *Eulota*, and also *Chiton*—the liquid was drawn toward the animal just as a wave passed over the opening in the plate, and then returned to its former level immediately after the wave had passed. This shows that the wave exerts a suction and must therefore be a concavity. The *Chiton* foot seemed to give the greatest amount of suction of all the mollusks tried, a difference in level of 3 to 4

mm. being observable. In all other cases a difference of at least 1 mm. could be seen with the exception of *Eulota* where the waves passed in such rapid succession that only a slight movement of the liquid could be detected.

#### SUMMARY

1. Three types of locomotion may be added to Vlès ('07) and Parker's ('11) classifications of gastropod locomotion, viz. (1) lateral, (2) diagonal, and (3) composite.

2. Cilia are the means of pedal locomotion in *Marginella*, *Haminea*, and *Bulla*.

3. *Chiton* and *Fissurella* are able to move backward without reversing the normal direction of their pedal waves.

4. Pedal waves can be shown by the use of a manometer to be areas of suction and therefore concavities.

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# A FURTHER CONTRIBUTION TO THE METAMORPHOSIS OF AMPHIBIAN ORGANS

## THE METAMORPHOSIS OF GRAFTED SKIN AND EYES OF AMBLYSTOMA PUNCTATUM

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FIVE PLATES AND THREE TEXT FIGURES

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## I. INTRODUCTION

In a series of experiments performed on larvae of *Salamandra maculosa* in 1910, 1911 and 1912,<sup>1</sup> the writer studied certain factors involved in the normal metamorphosis of the Amphibian eye. It was shown that eyes taken from an old larva and grafted to a young one, cannot metamorphose unless the host metamorphoses. The metamorphosis of the old eyes, therefore, was delayed as compared with the time of metamorphosis of their eye-mates left on the old larvae from which the grafted eyes were taken. In one case in which the new host metamorphosed two months later than the animal from which the grafted eye was taken, the metamorphosis of the grafted eye also occurred two months later than that of its eye-mate. Furthermore, it appeared that the grafted eye must metamorphose at the time that the eyes of the host metamorphose. When one eye of a young larva was grafted to an old larva, the metamorphosis of the young eye was accelerated just so much as to occur simultaneously with that of the host's eyes. In this way it was possible to accelerate metamorphosis of the grafted eye by two to six months. From these experiments, the following conclusion seemed justified; that metamorphosis of the eye is dependent upon the presence of a particular factor without which metamorphosis cannot occur, and that this factor cannot be produced by the eye itself, but is developed in another part of the animal's body whence it is furnished to the eye.

When however, very old larvae such as were leaving the water one or more days after the operation, were one of the two components (host or graft) in these grafting experiments, the results differed considerably from those described above. When one of these very old larvae (called 'Larvenendstadium' in the first publication) was the host the young grafted eye metamorphosed at a later date than the host and furthermore, when such an old eye was grafted on a young larva it metamorphosed long before and independently from the host. Though in these 'Larvenendstadien' the morphological change corresponding to meta-

<sup>1</sup> E. Uhlenhuth, 1913 a.

morphosis was not developed at the time of operation, the factor necessary to its production was apparently already contained in the eye. The young eyes grafted to the old animals on the other hand, did not metamorphose simultaneously with the host, because one or two days of action of this factor is apparently not sufficient to produce metamorphosis.

Thus, in the case of the eye of *Salamandra maculasa* the existence of a definite factor involved in the metamorphosis was shown. It was found (1) that this factor cannot be produced by the eye itself but originates in the body of the host, the exact part being unknown; (2) that it must act a certain length of time in order to start the process of metamorphosis and (3) that metamorphosis will occur if once induced by this factor, even if the organ is removed from the further influence of the action of the factor.

The fourth characteristic of the metamorphosis factor could not be precisely determined as the number of experiments was not sufficiently large. It was observed that eyes of salamander larvae also metamorphosed when grafted to larvae of Triton. These experiments suggested that the metamorphosis factor is a non-specific agent, but owing to the small number of experiments no comment was made at that time.

The first three characteristics however, were observed with such regularity that they were considered to be definite rules of the process of metamorphosis and apt to throw some important light on this phenomenon. In order to justify this opinion it was considered particularly necessary to examine several of these organs, the metamorphosis of which is most prominent and to find out whether the metamorphosis of these organs is subjected to similar or identical rules as the metamorphosis of the eye. If this were so, one would be justified in thinking that one single factor governs the metamorphosis of all these organs and that a common center in the body responsible for the development of this metamorphosis factor could be found.

It was from this idea that Kornfeld's experiments on the metamorphosis of the gills originated. By grafting the gills in a similar way as the eyes, it was proved that also in the meta-

morphosis of the gills a metamorphosis factor is involved, which possesses the first characteristic of the factor governing eye-metamorphosis. No experiments were made to examine the three other factors.

In 1913, several months after the author's first publication on the subject in question, Weigl's paper on homoplastic and heteroplastic transplantation of the Amphibian skin appeared. Some of his experiments were made in exactly the same manner as the author's eye-grafting experiments. To the facts obtained by these experiments we will have to refer later on. The conclusions however, which Weigl pointed out in a chapter on metamorphosis, were entirely contradictory to our results. From his experiments Weigl formed the opinion that the metamorphosis of the skin is a process of absolute self-differentiation of this organ (Roux)<sup>2</sup> while we found on the contrary, that the eye is unable to metamorphose unless it is furnished by the animal's body with a certain factor or agent, which alone can induce metamorphosis of the organ. If the conclusions of Weigl were right, the theory that metamorphosis of the organs is enacted from a common center in the body would certainly lose much of its interest and probability.

Meanwhile however, Gudernatsch's experiments on feeding tadpoles on thyroid, the first results of which were reported only a short time before the writer had finished his first group of experiments, have been repeated by several authors (Romeis, Kahn and others) and it is certain that thyroid substance can induce metamorphosis of the whole animal within a relatively short time. This again suggests that in normal metamorphosis the development of all organs, which take part in it, is governed by one center and that metamorphosis of these organs is not a process of self-differentiation.

Furthermore J. Loeb, following the ideas of Sachs, in a recently published book, has developed a theory according to which the flow of certain substances plays a very important rôle in the development of the organs. A large number of facts are quoted

<sup>2</sup> R. Weigl, p. 620.

which show that the mode of distribution by this flow of the substances determines the development of a certain structure at a certain place.

And finally, certain experiments are in progress and have been partly reported (Adler, Smith, Allen, Hoskins) by means of which one single organ (Hypophysis, Thyroid) has been removed from the Amphibian body, and it seems that the absence of this single organ actually prevents or modifies metamorphosis in some way.

From all this it seems that a study of the process of metamorphosis of the skin gains renewed interest and that the demonstration of a metamorphosis factor acting on the skin in a way similar to that of the metamorphosis factor involved in the metamorphosis of the eye and the gills, would be of great importance. For this reason a series of grafting experiments on *Amblystoma punctatum* and *Amblystoma tigrinum* were performed in the late spring of 1916. Special attention was paid to the metamorphosis of the grafted skin but in several cases the metamorphosis of the eye was also watched.

## II. CERTAIN MORPHOLOGICAL CHARACTERISTICS OF THE METAMORPHOSIS OF THE NORMAL SKIN AND EYE IN *AMBLYSTOMA PUNCTATUM*

### A. SKIN

As can be seen from the foregoing pages and will better be seen from the next chapter, the method of these experiments is such as to compare the skin of different individuals as to the time at which metamorphosis occurs or as to the rate at which development progresses. This cannot be done unless we have certain definite characteristics at hand which mark the time of the entrance of the skin into each stage concerned in this comparison. Therefore it is necessary to outline here briefly the course of the development of those characteristics which we have chosen for the purpose of indicating the entrance into certain developmental stages and to signify what characteristics we have decided to be our indicators.

It is well known that during metamorphosis the Amphibian skin is subjected to a large number of changes, some of which concern only the histological structure, while others are of such a nature as to alter the external aspect of the organ also. It is clear that we could not use the first ones, as the nature of our experiments does not permit of killing the animals for examination. Among the external changes only the development of the permanent coloration of the skin was used. As we shall see it is possible to discern a number of definite stages within the process of working out of the skin colors. The development of each of these stages may, and according to what has been said on page 1 of this paper actually does correspond to certain more or less complicated internal physiological processes, which are visible neither to the microscope nor to the naked eye; by means of these processes a number of internal changes may be effected, the final expression of which are those morphological changes with which alone we will be concerned in this paper. The nature of the internal changes connected intimately with the production of the pigments may be important and so far as the gathering of a certain kind of chromatophores at certain places into single spots is concerned seem to be of particular interest, but need not be discussed here. Concerning the action of the physiological processes, certain features of their nature have been disclosed by experiments similar to those to be reported and these are described in former papers concerned with these experiments.

Before entering into a description of the development of the skin coloration it should be mentioned that among the American species of Salamanders available for our experiments, there is none in which the development of the skin shows such a definite step as is the appearance of the yellow spots in the European species *Salamandra maculosa*. In *Amblystoma punctatum* the development of the permanent skin patterns of the adult is much more continuous and gradual; nevertheless as we will see it is possible to divide the development of the yellow spots of the adult animal into a number of well defined developmental stages.

The first color stage of the skin prevails during the largest part of the larval period; it is characterized by a more or less uniform pigmentation of the entire skin except the skin of the belly, which however does not enter into our experiments. This uniformity is produced during the first days of life by a continuous layer of a yellowish pigment which is not contained in chromatophores, but exhibits a purely diffuse character. Soon after the animals are hatched, melanophores appear scattered more or less evenly throughout the skin of the head, back and tail. With the gradual increase of the number of melanophores the skin continually darkens without losing the uniformity of its color. No definite patterns are worked out on the skin during this period, which shall be referred to as 'even yellow brown' in the following chapters. Of course, there are many variations to be observed corresponding with individual differences as well as with differences in the treatment of the animals and age of the animals; but none are definite enough to play any role here. Variations from a very light to a very dark shade usually correspond with progressing age and also with similar differences of the color of the background on which the animals are kept, with the intensity of light and the differences in temperature. Grayish, reddish or greenish tints may be intermingled but without changing the uniformity of the coloration. Figure 1 indicates this stage; it shows a larva of *Amblystoma punctatum* about six months old, kept in a dark room at 15°C. and fed on worms. The same stage may be seen from figure 13.

The next stage is the 'network.' It is characterized by the appearance of a yellowish or greenish network on an even yellow brown background. It also shows great variations: it may be very dense or very loose; it may be produced by either fine and numerous yellow streaks or by fewer and broader stripes and blots. It may be of intense yellow or of a greenish color. Similar factors to those mentioned in the last paragraph seem to produce these variations and we shall deal with some of them in another paper. Here it is important to mention that the network can be sharply defined as soon as it appears, though considerable time is required to develop all of its characteristics

definitely. It always develops while the animal is still in the larval period. Figure 2 pictures the network as it appeared on a larva six months old, kept in a dark room at an average temperature of about 15°C. and fed on worms.

The next stage may begin while the animal is still in the water and under these circumstances indicates that the animal will soon leave the water, or it may begin after the animal has left the water. At any rate, it always begins about this period, and the time of its appearance and further development corresponds to the period in which the animal as a whole undergoes those processes which are generally called metamorphosis in the Amphibians. (See Chapter III, p. 251.) For this reason this stage is considered here as corresponding with the metamorphosis of the skin. The beginning of this stage is indicated by a general contraction of the network, by means of which it loses its anastomoses and appears in the form of isolated spots or blots, a phenomenon which is—within the limit of a few days—well defined in respect to time. But the development of the definite yellow spots as they appear on the adult animal, occurs very gradually in most animals requiring about seventeen to twenty-one days and in some exceptional cases much more. After the animal has left the water, all colors soon appear quite dim in consequence of the development of relatively dry and dull layers of horny cells lying on the surface of the skin and unshed for a long time. During this period, the spots decrease in size and change their shape, assuming a round form in the majority of animals. Many spots disappear. This stage was called "Separation of the network." Unfortunately no photograph was taken of a worm-fed larva entering this stage. In its place a photograph of a thymus-fed animal kept at a temperature of about 25°C. is shown here (fig. 3); it was taken a short time after the separation of the network had started. Although there are certain differences between the color of thymus-fed and worm-fed animals, the stages of development show the same essential features in both cases. The day before this animal was photographed, 102 yellow spots were counted on the right side of the



animal, two and one-half months later, when the animal was fully metamorphosed, only 32 spots were left.

Figure 4 shows the separation of the network at a later period on a worm-fed animal which left the water eleven days before it was photographed and painted. This picture also shows that the development of the spots on the tail has progressed more rapidly than on the body. We shall refer only to the spots on the body and head.

The last stage which we call 'black' is reached when the colors of the spots and background have fully cleared up. It corresponds with the termination of metamorphosis. The background then appears black and the spots are of a varying bright yellow; the colors also are more vivid because the surface of the skin is now glossy instead of dull. The transition from the third into the last stage occurs very gradually and no definite line can be drawn between them. Figure 14 shows a fully metamorphosed animal.

In the following text the terms 'cinnamon' and 'brown' refer to the color of the background after the animal has left the water. In the beginning it very often assumes a reddish brown color which is called 'cinnamon'; then this color changes into a greenish black-brown which is called 'brown'. By getting gradually darker and darker this color becomes 'black.'

It should also be mentioned that the rest of the skin in which the network and the spots seem to be imbedded, is called "Background."

Finally it must be said that size and shade of the yellow spots varies greatly as we shall see later on, when we shall deal with this fact. One exception should be mentioned here. A very small percentage among a certain number of animals does not develop any spots at all on the back, or only very few of small size and of a faint greenish color. They seem to consist of a number of tiny yellowish pigment dots, which attach a loose aspect to them; they are called 'dotted spots.' In such animals the rate of development of the background is more rapid than the development of the yellow spots; at a similar stage of de-

velopment of the background in other animals the spots would have been better developed than in the animals referred to.

The four stages which we have found during the development of the skin are (1) even yellow brown, (2) network, (3) separation of network and (4) black; the third one, because of its simultaneity with the general metamorphosis of the whole animal, has been considered to directly correspond with the metamorphosis of the skin. But since the rate of development of the other stages and particularly also of the second stage has been found subjected to the same laws as that of the third stage, there will be no need of emphasizing this artificial distinction when we discuss the general laws to which the development of the skin appears to be subjected. The second and the third stage together constitute the phenomenon of the working out of one typical characteristic of the adult, i.e., the yellow spots, which is the first step of this stage.

#### B. EYE

In the eye also certain changes of its pigmentation were used as a mark of the entrance into metamorphosis.

In the beginning of the larval period the eye shows, when examined with the naked eye, a continuous rather light yellow ring, which corresponds with the iris and contains no black pigment. The color of this ring darkens with increasing age but no black pigment forming interruptions between the yellow parts of the ring are visible to the naked eye. Some time before the larvae leave the water the ring becomes narrower and finally forms a distinct and narrow but still continuous line around the pupil. Several days after the animal has left the water, the ring undergoes a distinct and very sudden change; the yellow line breaks up into numerous yellow and black dots. After this there occurs a very gradual change, which stretches over a long period; while the black dots become larger, the number of yellow dots decreases until finally pupil and iris form one black circle on the outer margin of which a small number of yellow dots are scattered. In some individuals no dots remain while in others yellow dots are present for several months.

Such yellow dots may be detected occasionally even in adult individuals.

On examination with a high power binocular lupe the phenomenon described above appears somewhat different. From the beginning a small number of contracted melanophores can be seen scattered throughout the yellow iris. The darkening of the iris is not due so much to an accumulation of melanophores the number of which increases very little, but to the appearance of orange or reddish yellow blots. In these blots the blood can be seen circulating while in the early stage the blood circulation could not be seen. It also seems that some of the yellow chromatophores themselves have become somewhat darker yellow. In the very early stages a division into two sections can be seen—an inner narrower section and an outer wider section. The inner section does not contain any melanophores at all and appears even under a weak magnification of the microscope as a continuous ring of pure yellow pigment (fig. 5). This division into two sections later on becomes more accentuated as the reddish yellow blots only appear in the outer section. Finally, in the outer section the amount of darker pigment increases and large parts of it appear brown while the inner section still remains without melanophores. It is at this time that to the naked eye the ring seems to have become narrower since the outer section has become black to the naked eye. At the time that the naked eye sees the breaking up into black and yellow dots, the yellow chromatophores of the inner section have started to loosen up and form—by means of their anastomosing processes—a fine network through the meshes of which the black epithelial layer of the iris seems to become visible. Soon the yellow chromatophores become fewer and cease to anastomose with each other. This process and a gradual decrease in the number of isolated yellow chromatophores correspond to the decrease in the number of yellow dots seen by the naked eye; each yellow dot corresponds to one single xanthophore (fig. 6).

The stage of breaking up into yellow and black dots will be assumed to be the beginning of the eye's metamorphosis.

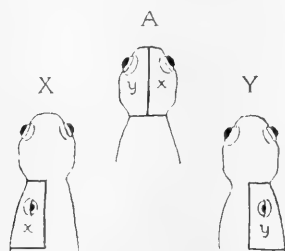
## III. THE EXPERIMENTS

A. HOMOPLASTIC TRANSPLANTATION BETWEEN LARVAE OF  
AMBLYSTOMA PUNCTATUM*1. Material and method*

The general method of these experiments should be to remove one piece of skin from the larva just before the factor inducing metamorphosis is developing in the body, and to graft the piece to another larva in which this factor will not develop for a long time. The animal from which the skin graft is taken should metamorphose long before the piece of skin which was grafted onto the young host would metamorphose, provided the metamorphosis of the skin follows the same rules as that of the eye and gills. Grafting of a young skin to an old larva in which the metamorphosis factor is just to develop should give a result opposite to that of the first mentioned experiment; metamorphosis of the skin graft should be accelerated and should occur before the animal from which the graft was taken would metamorphose.

In order to use this method, it is necessary to have on hand larvae whose age varies sufficiently to make it certain that with a younger animal also, an earlier stage of development will correspond, which relation according to a well known experience exists in Amphibians, only if the individuals differ in age by months. Since we used larvae whose age varied only by a few days we had to modify our method. The new method was based on the fact that in a lot of animals hatched on the same date, the rate of development varies, sometimes to the extent of several months, in spite of apparently the same treatment. It was hoped then, that of two individuals chosen at random, one might develop more rapidly than the other; by increasing the number of pairs there would also be an increase in such pairs whose individuals would develop unequally. Thus each experiment consisted of two animals, X and Y (text fig. *a*), to each of them one of the two eyes of a third animal A and the skin of the corresponding half of the head of animal A was grafted. If

left with animal A the skin of both halves of the head would have metamorphosed at the same time; namely: at the time when the factors necessary for the metamorphosis of the skin after they have developed in the body of animal A, and have been carried into the skin, have acted in the skin a sufficient length of time to cause the physiological changes necessary to produce the morphological changes of the skin described in the preceding chapter. But removed from the animal A and separated from each other—one half having been grafted to X, the other half to animal Y—they would not metamorphose simultaneously with each other but simultaneously with their respective new hosts and hence at different times; provided of course, that



Text figure a

metamorphosis of the skin followed the principles observed in the metamorphosis of the Amphibian eye and gills.

To use the skin of the head for grafting offers a special advantage. As pointed out in Chapter II, in studying metamorphosis of the skin of *Amblystoma punctatum* we must choose some definite characteristic of the skin. For certain reasons explained in Chapter II the appearance of the yellow spots was found to be the most convenient time-mark. In the larvae, however, where of course no spots are developed yet, it is impossible to foresee on what parts of the skin of the body such spots will develop and as the distribution of the yellow spots is subjected to great variation there would be little chance of having selected for grafting a piece of skin that would finally develop a spot. But this is different for the head, as in the majority of animals one or two yellow spots will develop on the skin above

both sides of the lateral 'os occipitale.' Among 83 individuals for instance, which were examined in this respect, only 16.9 per cent did not have any spot on the right side (only this side was counted) of the caudal end of the head, while 83.1 per cent of the animals had developed one or more spots. Therefore, if the skin of the head is used, the chance of obtaining yellow spots in the graft is about 83 per cent and one is also prepared at the time of metamorphosis to look for these spots on the rear end of the skin graft, provided that the skin be grafted in such a way that its front and rear correspond to cranial and caudal respectively of the new host. In the experiments the actual number of grafts which developed spots on the rear end was much smaller than 83 per cent, the reason for this being that when the grafts were taken from the larvae, they did not always include the area where the spots mentioned above are located.

The technique of operation was exactly the same used in former experiments and may be learned from previously published papers on these experiments.<sup>3</sup> It should be mentioned however, that while front and rear end of the graft were left unchanged, each piece of skin was turned upside down on its new host, as can be seen from the sketch in figure 1. In the first six experiments the animals were kept in an icebox at 0° to 3°C. before operation. All other animals were kept in a dark cool-room at a temperature of 14° to 18°C. before operation. Immediately after operation each animal was placed in a petri dish, the bottom of which was covered with moist filter paper and a thin layer of water, and placed in a dark incubator at 16° to 18°C. for about twelve to sixteen hours. Then each one was put into a white glass jar holding about 2000 cc.; the jars were filled with tap water and placed in a dark cool-room at an average temperature of 15°C. where the animals were kept permanently.

When the animals should be taken out of the water they show a rather definite and uniform behavior. First they come to the surface of the water in order to change the air contained in the

<sup>3</sup> E. Uhlenhuth, 1912, p. 735.

lungs; soon after this they begin to float permanently on top of the water and do not take food for several days. The gills and fins start to reduce in size and then the animals suddenly shed their skin and in the short space of a day or over night, the gills are reduced to mere small filaments. If the animals are not removed from the water at this moment they soon become stiff from asphyxiation, but may recover if taken out before dead. When removed from the water each animal was transferred into a glass jar of the same size as those used for the larvae, but the bottom of the jars was covered with a piece of moist filter paper and a small amount of water. The jars were kept closed all the time by means of plate-glass. In the following text this condition will be called 'land-condition.' This equipment was not changed until the beginning of 1917, when the filter paper was replaced by gravel.

As long as the animals were larval they were fed on *Tubifex* thrown into the water. After metamorphosis earthworms were fed. Though most of the animals after they had been metamorphosed commenced to take readily pieces of earthworms from a pair of forceps, they later on stopped doing this after they had been neglected for some time and had to be fed forcibly by stuffing the pieces into their mouths. It appeared however, that this method of artificial feeding was not satisfactory and many of the animals were lost. It should be mentioned that by feeding the animals regularly they easily learn to take even whole earthworms from the forceps and there is no difficulty in raising Salamanders to full size in this way as we have experienced in other experiments.

Forty-six larvae of *Amblystoma punctatum* were operated upon in the manner described above, an odd number and the consecutive even number making one pair X and Y, each of which carried on one of its shoulders a piece of skin and one eye taken from the head of a third animal A (fig. 1). The animals used for these experiments hatched between the 3rd and 8th of May, 1916 from eggs collected by Mr. R. Deckert of Bronx Park in swamps near Whiteplains, N. Y., during the spring of 1916. All three individuals of one pair (A, X and Y) were

usually hatched on the same day. Whether or not their origin was from the same mother was not noted. The operations were performed from the 6th to the 22d of July, 1916. The results of this series (Series XXV) are described in the following pages.

## 2. *The Results*

*a. The metamorphosis of the skin grafts.* Not all 23 pairs gave conclusive results, as was to be expected from the character of the experiments. In six pairs one of the two individuals died before metamorphosis; in six other pairs the development of those characteristics used as indicators of metamorphosis showed some irregularities which prevented final conclusive results. In the remaining eleven pairs the result was conclusive. According to this the experiments may be divided into three groups.

1. In six pairs one animal died a short time after the operation, the remaining animal was nevertheless watched and it was observed that with one exception (experiment 13) where no result was reached at all, the development of the graft proceeded simultaneously with the development of the host.

In experiment 1 the animal was set on land-condition on September 1, 1916;<sup>4</sup> the first greenish spot on skin graft appeared on September 2; the network of the host was separated into greenish spots on September 5. In host and graft the spots assumed more definite outlines on September 18 and turned yellowish on October 2; on October 3, host and graft appeared cinnamon after having become gradually darker and darker. Both were still darker on October 31; no further examination could be made as the animal died.

In experiment 3, host and graft both turned gradually darker and darker until the animal was set on land-condition on September 7. On September 9, host and graft had developed green spots.

<sup>4</sup> All observations and notes from the end of July to October 2 were made by Mr. N. Anderson with the aid of a detailed plan given to him for this purpose. All observations and notes previous and subsequent to this period were made by the author himself. On October 3 the results found at this time were compared with Mr. Anderson's notes and were found to be in accordance with them.



In experiment 6 the yellow network of the host had formed on September 11, while on graft no network ever was developed. The animal was set on land-condition on September 23 and October 9 the network of the host had separated into greenish spots and simultaneously on graft two yellowish spots had come out.

In experiment 7 the yellow network of the host appeared on August 28, while on graft there was never any network formed. On August 30 the network of the host was already separated into greenish and yellowish spots and on graft, 5 small spots had formed; the animal was transferred to land-condition the same day. On September 5 host and graft were already very dark and cleared up gradually until they were finally black and their spots bright yellow.

In experiment 13 the host was one of those animals which for some reason do not develop any spots at all on the back or form only after a long time a few faint and small greenish spots, consisting of very loose, tiny greenish or yellowish pigment dots. For this reason this experiment appears to be of very little value in connection with the problem of metamorphosis. Since we will have to come back to this experiment when other problems will be at issue, it may be briefly outlined here.

On August 28 the graft had developed two light yellow spots, while the host showed only a very faint greenish pigmentation spread over the yellow brown background. After the animal was set on land-condition (September 1), host and graft turned darker and darker, but while the graft still had two large greenish spots, the host had remained in the network stage and its network was of such a faint and indefinite character that it could not be traced exactly nor ascertained whether or not it was isolated. The spots of the graft turned bright yellow on September 11 while on the host's body the first greenish spots were not recognized before September 18 and did not become distinct before September 23 (three weeks after the animal had left the water). The body of the host never developed more than a few small greenish dotted spots, but turned black simultaneously with the graft, whose spot finally disappeared.

In experiment 18 host and graft had become quite dark yellow brown on August 28 and on this day both had developed greenish spots. After the animal was set on land-condition (September 1) host and graft turned continuously darker and their spots grew more yellow. Finally, when the spots of the host began to brighten up the graft lost its spot, but its background changed simultaneously with the host into a clear black.

Though in five of these experiments—the sixth will be referred to later—the graft metamorphosed simultaneously with the host, the result is of course, not conclusive, as none of these grafts was controlled by the other animal of the pair, which carried the other half of the skin of the head of animal A; it could therefore be possible that the animal A from which the grafts were taken would have metamorphosed—if living—at the same time as the respective new hosts. Hence the time of metamorphosis in the grafts might have been determined by the animals from which the grafts were taken and not by the new hosts. On the other hand, the results obtained by these experiments are not contradictory to the idea that metamorphosis of the grafted organs is governed by the body of the host and might be even explained on the basis of this rule in case a number of other conclusive experiments could be shown to prove the existence of such a rule.

2. In 6 pairs (experiments 15 and 16, 19 and 20, 23 and 24, 29 and 30, 35 and 36, 37 and 38) both animals lived long enough to metamorphose, but the results were not conclusive for some other reasons. In some of them either the graft or the host did not develop spots, while in others both hosts metamorphosed at the same time, and still in some other experiments the observations made during the development of the skin were not complete enough.

In pair 15 and 16 the graft of experiment 15 did not develop either network or spots; nevertheless it developed simultaneously with the host as indicated by the notes on the changes of the background, and after metamorphosis cleared up into black at the same time that the host's skin did. In experiment 16 the notes on the development of network and spots are incomplete

and as the animal died soon after being set on land-condition it was not possible to watch the process of clearing up.

In the pair 19 and 20 the grafts of both animals did not develop spots and also did not show any network, but in both animals the changes of the background progressed simultaneously. When the animals died (September 19) they were preserved in formalin; from the formalin specimens it can still be seen that in both animals graft and host are at the same stage of development. Unfortunately, as both hosts had developed at the same rate, this pair does not permit of any conclusions concerning the determination of the metamorphosis of the grafts.

In pair 23 and 24 both grafts were spotless pieces of skin. They nevertheless developed a network; but as both animals X and Y formed their network at the same time the grafts also developing simultaneously with their respective hosts, also formed the network at the same time and there was no way of knowing whether the formation of the network was determined by the hosts X and Y or by the animal A from which both grafts were taken. In experiment 23 the graft developed a little spot underneath the eye just at the same time that the host's network separated into yellowish green spots. This little spot soon disappeared. In experiment 24 however, no spots developed at all except a faint little patch of tiny pigment dots believed to be seen for only two days on the hind corner of the graft, about 1 month after the host's network had separated into spots. It is however impossible to draw any conclusions from these 2 experiments.

In experiments 29 and 30 the graft of animal X (experiment 29) never developed any spots, while the graft of animal Y never formed a network; so there was no way of comparing the two experiments with each other. It may be mentioned however, that in experiment 29 the network of the host appeared simultaneously with that of the graft (August 14) and that in experiment 30 host and graft formed their spots at the same time.

In the pair 35-36, network and spots of host and graft were formed simultaneously in experiment 35, but the graft of experiment 36 was a piece of skin which did not develop spots, the

notes about the formation of the network of experiment 36 are incomplete.

In experiment 37 where the spots of host and graft formed simultaneously, the notes about the network are missing, while in experiment 38 where the network was formed simultaneously in host and graft, the latter never developed any spots, thus making comparison impossible.

These 12 experiments do not permit of any conclusions as to whether the metamorphosis of the skin actually is determined by the body of the host, neither do they prove anything to the contrary.

3. Twenty of the remaining experiments forming ten pairs were so complete as to correspond in every respect to the plan designed before the experiments were started and as will be found, these experiments actually support the idea that was outlined in the first pages of this article. The eleventh pair should rather have been placed in the second group but for some reasons, which will be understood later on it was deemed to be convenient to describe the experiments 11-12 under this group.

For each pair a brief abstract from the records collected in the progress of the work is given here.

In experiments 21-22, the hosts developed in the beginning at the same rate, and so did the grafts. Later on experiment 22 got somewhat ahead and developed the spots three days before experiment 21 and consequently the graft of experiment 22 also developed its spots three days before the graft of 21 did, though both grafts were taken from the same animal. We must admit, however, that not too much weight can be laid upon this result; in the first place the animals were examined only twice a week and the appearance of the spots on the graft of experiment 21 might have been reported three days later than they actually formed; in this case the spots would have formed simultaneously with the spots of the other graft. Secondly it takes several days as a rule for the spots everywhere on the head and body to become isolated, so that some may appear isolated a few days earlier than others even in the same animal; in this case the difference between the grafts might have been caused not by

their hosts but by the animal A to which they both originally belong.

In both grafts the spots from the very beginning of their appearance were much brighter than the spots of the host; this however, is in no way due to a difference between the rate of

DATE	EXPERIMENT 21	EXPERIMENT 22
August 7	Host and graft even light brown	Host and graft even light brown
August 14	Host and graft have developed a greenish network	Host and graft have developed a greenish yellow network
August 14	Land-condition	Land-condition
August 30		On host network separated into dark green spots; on graft 3 yellow spots have formed, 2 of which are very bright
September 2	On host network separated into dull yellow spots; on graft 3 very bright and large yellow spots have developed	
December 14	Host and graft have become black step by step. Only 1 spot has remained on graft, which still is far brighter than any of the spots of the host; it is almost orange yellow, while the spots of host are light yellow. The graft's spot is 3 times as large as any of the spots of the host	Host and graft have become black step by step. Only 2 spots have remained on graft, 1 of which is twice as large as any of the spots of the host. Both are still much brighter than the spots of the host and almost orange yellow, while the spots of the host are light yellow. But there is 1 spot in front of the graft on the head of the host which is as bright as the spots of the graft and of the same color as these spots

development of host and graft but must be explained on the ground of a specific difference existing between the spots of animal A from which both grafts were taken and the spots of the animals X and Y; only this assumption can explain the fact, that the same characteristics appeared in both grafts alike and were retained permanently, since the spots were even more unlike

the host's spots after the animal was fully metamorphosed (figs. 7 and 8). We shall return later to this phenomenon. It is more difficult to understand the origin of the orange yellow spot in front of the graft of experiment 22, as the history of this spot is missing. But it seems certain that this spot either belonged to the graft or was formed at least under its influence and with the aid of the graft's yellow chromatophores. In favor of the suggested origin of this spot is the fact that among a very large number of metamorphosed animals which did not have pieces of skin grafted from other individuals, such a wide and very distinct difference in the color of the spots was never observed.

DATE	EXPERIMENT 41	EXPERIMENT 42
August 7	Host and graft even yellow brown	Host and graft even yellow brown
September 5	Host and graft have developed the greenish network	Host and graft have developed the network
September 9	Land-condition	
September 11	Host and graft becoming darker; in both network has isolated into large irregular green spots	
September 16		Host's network separated into spots in several places; on graft 2 greenish spots are formed

In experiments 41-42, the grafts developed their network simultaneously with each other, as also did the hosts, but the host and graft of experiment 41 developed spots five days earlier than host and graft of experiment 42.

In experiments 25-26, the development of the network on the grafts occurred simultaneously with their respective hosts, but not simultaneously with each other: Hence just the opposite occurred of what would be expected if the rate of development of the grafts were determined by animal A, to which both grafts originally belonged. In experiment 26 the first trace of the network appeared seven days earlier than in experiment 25. In

experiment 26 the graft developed all other characteristics at the same rate as its host; in experiment 25 the graft was a spotless piece of skin and cannot be compared further with the host

DATE	EXPERIMENT 25	EXPERIMENT 26
August 7		The first traces of a light yellowish green network have appeared on host and graft
August 14	Host and graft show first traces of a green network	
August 22	On the host the network assumes a more distinct character, while in graft it has become less conspicuous	
September 5	In the host network has started to separate into yellow spots, while in graft only a faint trace of network is left and no spots are formed	
September 8	Land-condition	
September 9	Host and graft cinnamon	
September 11		The network of the host has differentiated distinctly from the brown background and has assumed a yellowish green color. On the graft the network has spread its grayish green pigmentation and left only 2 brown lines of the background uncovered
September 15		Land-condition
September 25		On host network has started to separate into large green spots, on graft 1 spot has formed in hind-corner
September 28		Host and graft cinnamon
October 7	Host and graft dark cinnamon	Host and graft very dark cinnamon

with regard to spot development, but its background changed simultaneously with the host.

In experiments 31 and 32 the network as well as the spots in both grafts developed simultaneously with their respective hosts,

DATE	EXPERIMENT 31	EXPERIMENT 32
August 14	Host and graft show the first appearance of a greenish network	
August 28		On host and graft a grayish network begins to appear; on host it is more of a greenish shade
September 13 September 14		Land-condition On host and graft yellow spots have separated from network
September 17 September 23	Land-condition On host large irregular green spots have formed from the network; on graft 1 large greenish spot has developed right behind eye	

but not simultaneously with each other. In experiment 31 the network of the host and graft was formed fourteen days earlier than in host and graft of experiment 32, while the spots separated nine days later than in experiment 32. This result can be understood if we assume that the development of network and spots of the grafted skin was determined by the new hosts.

In experiments 9-10, no notes were made on the development of the network. The spots of host and graft developed ten days earlier in experiment 10 than in experiment 9.

In experiments 39-40, the network of the grafts again developed as if the rate of development would be determined by the respective hosts. In graft and host of experiment 39 it

DATE	EXPERIMENT 9	EXPERIMENT 10
August 28	Green spots appear on host and graft Land-condition	First yellow spots appearing on host's skin and 1 on rear end of graft
August 31 September 7		Land-condition
September 9		



appeared fourteen days earlier than in experiment 40, and in experiment 40 it became distinct twelve days after the isolation into spots had started in experiment 39. The development of the spots of experiment 40 occurred simultaneously in host and graft; in experiment 39 the graft was a spotless piece of skin and comparison of the development of the spots was impossible.

DATE	EXPERIMENT 39	EXPERIMENT 40
August 14	On host and graft first traces of greenish network have developed	
August 28		On host and graft first traces of a greenish network appear
August 30	On host first greenish spots have isolated from network. On graft only in center a part of the greenish network still present	
August 30	Land-condition	
September 6	On host network everywhere separated into large irregular green spots. In graft network disappeared, no spots developed	
September 11		It is only now that the green network of the host and graft has become distinct
September 15		Land-condition
September 23		On host and graft network separated into irregular greenish spots
December 21	Host's spots almost orange-yellow, graft has no spots	Host's spots almost orange yellow; graft with 2 light yellow spots

This case is perfectly clear as to network development. It developed simultaneously in each graft and its host, but twenty-one days earlier in graft and host of experiment 46 than in graft and host of experiment 45. The development of the spots however, seems confused at the first glance in both experiments. In experiment 45 the host developed its spots 11 days after the spots of the graft had developed. We have already mentioned

a similar case (experiment 13) and we will find a few more animals acting similarly. All individuals which developed their spots after the graft had developed them, showed one common characteristic when they were fully metamorphosed: they were almost bare of yellow pigment. The host of experiment 45 for instance, developed only very small and faint spots which lacked the usual compactness of the spots and gave only

DATE	EXPERIMENT 45	EXPERIMENT 46
August 7		On host and graft greenish network has developed
August 28	Host and graft have formed a greenish network	
September 12	Land-condition	Land-condition
September 14		Host's network has isolated into green spots; in graft yellow pigment dots, chiefly in center, have developed, but no large spots
September 21	Network of the host has not separated yet, but forms a greenish rather faint pattern, while in graft 1 yellow spot has formed in the rear end	
October 4	Host's spots appeared today	
October 31		Spots of the host bright yellow; graft spotless
December 26	On the body of the host (right side) there are only 3 small and faint light yellow dotted spots, while the spot of the graft is very bright and almost orange yellow	

the impression of an accumulation of little yellow dots on the black background. As their number (on the right side) was only 3 (fig. 9), the animal appeared almost uniformly black. We believe therefore that in this case a factor whose nature will be explained later on, interfered with the metamorphosis factor. In experiment 46 the graft did not develop any real spots, but only a few small dots which appeared simultaneously with the

spots of the host, but later on disappeared entirely. In this case, however, it was impossible to decide whether or not this was due to the graft being a spotless piece of skin as this graft soon after metamorphosis assumed a pathological appearance; it became grayish and edematous and was gradually replaced by the surrounding skin of the host. Therefore the development of the spots in none of the experiments of this pair can be used in studying the problems of metamorphosis.

In experiments 43-44, the grafts developed the network independently from each other and simultaneously with their re-

DATE	EXPERIMENT 43	EXPERIMENT 44
August 28	On host and graft first traces of a greenish network have formed	
September 5		On host a greenish yellow network has started to form, on graft the network consists of a faint grayish green pigmentation
September 11		On host several yellow spots have isolated, in graft 1 yellow spot has appeared
September 13		Land-condition
September 30	Land-condition	
October 4	Host's network separated into dim yellowish spots, in graft some yellow spots are developing on rear end	
December 26	Host with bright yellow spots, graft has lost all its spots	Host with large yellow spots; graft has lost its spot

spective hosts, 43 forming the network of host and graft eight days before host and graft of 44. Both grafts finally became spotless, since the spots developed in the beginning disappeared later on. But these temporary spots were developed in both grafts at the same time as the spots of the respective hosts, in experiment 43, twenty-three days later than in experiment 44.

Experiments 27-28 are perfectly conclusive in regard to the development of the network, which was formed by both grafts simultaneously with their respective hosts, but appeared in host

and graft of 27, twenty-eight days later than in host and graft of 28. Concerning the development of the spots we find that

DATE	EXPERIMENT 27	EXPERIMENT 28
July 31		On host and graft a grayish green network has started to form
August 28	On the host a greenish network has started to develop; on graft a faint green network also is forming	
August 30	The network of the host has separated into bright yellow spots and from the network of the graft patches of yellow pigment dots have developed in the center of the graft, which are less bright than the spots of the host	
August 30	Land-condition	Land-condition
September 5		In host there are no spots, only
September 9		a network like greenish pigmentation while in the graft only a part of the network is left and in the center 2 distinct yellow spots have formed
September 14		In host the network is still present but several yellow spots around the skin-graft have finally developed
October 7	Host's spots still dim yellow, graft's spots disappeared	
October 12	Animal escaped	
December 18		Host's body almost without spots, since only 3 light yellow spots near the shoulder of right side and a few around the grafted skin of the left side have developed

the graft of 27 finally became spotless, but developed during metamorphosis a few temporary spots simultaneously with the host. In experiment 28 we have before us a case which is similar

to that of experiment 13 and 45. The network of the host did not separate when the network of the graft loosened up into spots, but needed five days longer for this process and even then had not entirely disappeared. When the animal had completed metamorphosis it turned out to be again one of those individuals whose skin is almost lacking in yellow pigment; only a few light spots had formed on each shoulder (fig. 10). This case will be discussed together with experiments 13 and 45 later on.

In experiments 11-12 we have a case similar to experiment 28 which will demonstrate particularly well the difference between the factor that is concerned in this kind of delayed development of the yellow spots and the factor which is involved in metamorphosis, for in this case both hosts were of this almost spotless variety and both behaved towards the grafts in a similar way. In addition to this, the grafts were taken from an animal whose spots were characterized by a particularly vivid and almost orange yellow coloration as shown by the fact that both grafts showed this feature in the same way. Since both characteristics are permanent even after metamorphosis (figs. 11 and 12) they apparently must be parts of the specific individuality of the adult animal, which of course, cannot be changed by the action of the metamorphosis factor, but on the contrary should be developed by this factor.

In the experiments mentioned until now the difference between the rate of development of the two grafts was rather slight, ranging from seven to twenty-eight days in regard to the network and from three to twenty-three days in regard to the spots. This was apparently due to the fact that the respective hosts did not show any greater differences. The last pair of experiments, however, will prove that the difference between the rate of the grafts increases in exactly the same way as the difference between the rate of development of the respective hosts increases and that one graft may metamorphose very much earlier than the other one as soon as one succeeds in accelerating metamorphosis of one of the hosts and in delaying metamorphosis of the other host a considerable length of time.

In this pair of experiments one animal (experiment 34) did not metamorphose at all, though it was kept under apparently

DATE	EXPERIMENT 11	EXPERIMENT 12
September 5	On graft 2 pale spots are developed. On host there are no spots as the network has not yet separated	In graft and host the network is formed.
September 8		Land-condition
September 9		The network of the graft has separated into 2 yellow spots, the network of the host has not yet separated
September 11	On graft 2 more spots have developed. On host very faint and fine yellow pigment dots appear occasionally	
September 13	Land-condition	Host's network separated into faint green spots; graft's spots bright yellow
September 20		In front of the grafted eye 1 bright yellow spot has developed on the graft
September 22	On the host several faint spots can just be traced	
September 23	The spot of the graft appears very bright compared with the spots of the host	
December 13	Host only with a few pale, very small dotted greenish spots, graft with 1 very bright, almost orange yellow spot	Host only with a few pale, very small dotted greenish spots (none on head and only 1 on right side of body). Graft with 2 pale dotted spots and 2 very bright, almost orange yellow spots, 1 of which in front of eye, cannot be determined now as lying on the graft, because the graft's borders have disappeared but its history shows that it developed on the graft

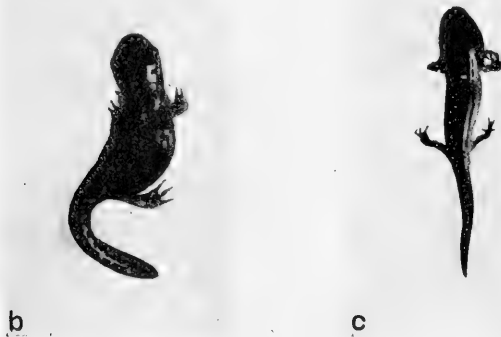
exactly the same conditions as experiment 33 and as all other specimens described until now. On January 10 it was subjected to another operation in consequence of which it died; at this time

the animal still did not show any signs of metamorphosis nor did its skin graft. Host and graft were of an even coloration and no spots or network had developed. Host and graft of experi-

DATE	EXPERIMENT 33	EXPERIMENT 34
July 19	Operated	Operated
August 7	On host and graft the first traces of a greenish network are already visible	Host and graft even yellow brown
August 28	On host and graft a distinct network has developed	
September 8	Land-condition	
September 11	Host's network separated into yellowish green spots; on graft 2 large yellow spots have developed which are brighter than host's spots	
September 25	Spots of the host greenish yellow; spots of the graft very bright yellow	
October 10	Photographed and painted in natural size	Photographed and painted in natural size
October 19		Photographed in four times the natural size and painted
1917		
January 10		Host and graft still even yellow brown (no network or spots), both being in larval condition
January 10		Dead (killed by a second operation).
January 30	Dead (preserved in formalin)	Preserved in formalin
February 18	Photograph of October 10 enlarged and painted with the aid of the notes, the formalin specimen and some living animals, which were similar in color to experiment 33	

ment 34 were in larval condition five months after the other graft—though taken from the same animal A as the graft of experiment 33—and its host had developed the first traces of the network and four months after they had developed their spots.

This will be illustrated best by the photographs and pictures which were made from both experiments. Both experiments were photographed in natural size on October 10. Text figure 2 shows experiment 33 fully metamorphosed and the spots developed all over the body. Behind the eye four spots are visible; two of them, lying behind each other in a line parallel to the longitudinal axis of the body, are only highlights produced by the reflection of the light on the black shining skin of the animal. The other two lighter spots are two yellow spots belonging to the graft. Just in front of the bulb of the grafted eye a third small spot is situated which also belongs to the graft. All these



Text figures *b* and *c*

spots were almost orange yellow, thus being distinctly different from the spots of the host; the two spots in back of the grafted eye were considerably larger than any of the spots of the host, which fact is well shown in the picture. Text figure 3 shows experiment 34 photographed on the same day as experiment 33. This animal was still larval at that time, as the gills are still present and no yellow spots are developed. In accordance with the condition of the host the graft, whose borders can be only partly distinguished in this photograph, has no spots also.

Nevertheless these photographs were not considered entirely convincing since they lacked detail, in addition to which they also could not demonstrate the rather minute details of the



eye which will be needed later. Experiment 34 was therefore photographed again on October 19, and this time in four times the natural size; the photograph was then painted carefully. This is shown in figure 13. The borders of the graft can now be seen quite distinctly, the graft is still the even color of a larva of *A. punctatum*, but is slightly darker than the host. The dark patch in front of the eye corresponds to a tumor-like elevation of the grafted skin produced probably by a proliferation of the epithelial cell, such as is often found in examining skin grafts of Amphibians on microscopic sections. The photograph of experiment 33 taken on October 10 was enlarged on October 19 but was not painted before February 18, 1917 (fig. 14) when the animal was already dead and preserved in formalin. In the period from October 10, 1916 to January 30, 1917 (the date the animal died) the two spots of the graft in back of the eye and particularly the spot in front of the eye, increased somewhat in size; the size of these spots in figure 14 corresponds to their size on January 30, 1917, as they were drawn from the preserved specimen. The picture (fig. 14) shows also that besides the difference in size there is a considerable difference in the color of the graft's and host's spots. When the picture was made the colors were painted according to the notes made during the observations of the living specimen, with the aid of other living animals whose color was known to have been similar to experiment 33, and partly from memory; but it is believed that the picture is in color at least, very close to the actual colors of the living specimen. In the preserved formalin specimen all spots are of course, much bleached, but nevertheless the difference between the color of the spots of the host and graft can be seen most clearly.

*b. The metamorphosis of the grafted eye.* As previously mentioned no special attention was paid to the metamorphosis of the eye in this series of experiments on *A. punctatum*. Only in one pair of experiments (33-34) described in the last chapter, the metamorphosis of the eyes was watched during part of the life of the animal, so as to give a fairly continuous series of observations. An abstract from the notes is given here.

DATE	EXPERIMENT 33	EXPERIMENT 34
September 8	Land-condition	Larval-condition
September 29	Today the grafted eye was examined for the first time, but the eyes of the host were not examined. The yellow ring of the grafted eye is broken up into black and yellow dots	Host's eyes as well as grafted eye still possess a plain, continuous yellow ring
October 17	Today the eyes of the host were examined also. In all three eyes, the two eyes of the host and the grafted eye, the yellow ring is broken up into yellow dots	The rings of all three eyes are free from any black pigment
December 19	In the iris of both eyes of the host a number of very tiny yellow dots are still present, while the rest of the iris has become black. In the iris of the grafted eye a small number of yellow dots are still present, the rest of the iris is black	The inner section of the iris of the grafted eye appears, even with a larger magnification of a binocular lupe to be a continuous yellow ring, in which no melanophores are detected. The outer section contains a large number of yellow chromatophores, which are darker than the other yellow chromatophores and more reddish, and contains several parts in its course which are made up by grayish-blue cells. Both eyes of the host are in exactly the same condition as the grafted eye
1917		
January 10		All three eyes still in the same condition as when last noted.
January 10		Killed by a second operation

From these observations it is evident that in experiment 34 the ring of the grafted eye was still unbroken, about three months after the yellow ring of the grafted eye of experiment 33 was broken up into black and yellow dots. The same relation existed between the eyes of the respective hosts. The condition of the grafted eye as it existed on October 19, may be seen in figures

13 and 14; this condition was only little changed, when the animal died. Hence, in experiment 34 metamorphosis of the grafted eye could not take place because the host did not metamorphose, while in experiment 33 the grafted eye, though belonging originally to the same animal A as the grafted eye of experiment 34, was forced into metamorphosis by its host at least three months earlier than experiment 34.

#### B. HETEROPLASTIC TRANSPLANTATIONS FROM LARVAE OF AMBLYSTOMA TIGRINUM TO AMBLYSTOMA PUNCTATUM

The object of these experiments was to ascertain whether or not organs of one species could metamorphose on the body of another species. Eyes and pieces of skin were grafted from *A. punctatum* to *A. tigrinum*. The experiments actually showed that eyes as well as skin are able to metamorphose even when grafted to another species, and that they metamorphose at the time that the host metamorphoses.

##### *1. Material and method*

The technique of operations employed in these experiments (series XXVI) was the same as that used for series XXV. The skin of one half of the head including the eye was taken off from a larva of *A. punctatum* and grafted to the region just back of the shoulders of a larva of *A. tigrinum*, after a piece of skin the size of the graft and a small amount of tissue of the *musculus longissimus dorsi* had been cut out from there.

The general method was somewhat different from series XXV, as the experiments were not arranged in pairs; the animal A from which the graft was taken being a different individual for each experiment. The left eye of individual A (*A. punctatum*) was grafted to individual X (*A. tigrinum*), X being the experimental animal which alone was kept alive and examined from time to time.

It was of course, impossible to compare the developmental stages of the grafts with the developmental stages of the host with such exactness as in series XXV, since the changes of the

organs of *A. tigrinum* are entirely different from those of the graft. Consequently only one definite step was left to which to refer the changes of the grafted punctatum organs, and that was the moment when the tigrinum host leaves the water. Doubtlessly, a certain amount of inaccuracy enters these experiments, for different individuals may show a different degree of development of some of their characteristics when they leave the water, a condition frequently noted during observation of a large number of Urodelan larvae. We shall see, however, that the difference in the dates when the hosts left the water is wide enough to establish a corresponding difference between the grafts and to make the relation between host and graft a definite one.

Furthermore, it must be said that it was not known exactly when the grafts would have metamorphosed if they had remained with individual A, for this individual had been killed and could not be used as a standard. But an approximate normal standard-time of the metamorphosis of the graft can be calculated from the records made about the time of metamorphosis of the entire stock of *A. punctatum* larvae from which our grafts were taken. According to these notes, from approximately 300 punctatum larvae all but about 10 metamorphosed before October 1, 1916. Four of the five grafts to be reported also metamorphosed before this time, together with their hosts; the fifth metamorphosed late in October and later than any *A. punctatum* larva of the entire series of experiments described in this paper. As the respective host showed a corresponding difference, compared with the other hosts, it seems justified to conclude that in all probability the time of metamorphosis of the grafts in this series (XXVI) was also determined by the hosts.

The material used as grafts was the same as used in Series XXV; all animals were hatched on May 6 and were in this case, the offsprings of the same mother. The tigrinum larvae were collected in the early part of July, 1916 in ponds on Long Island by Mr. Deckert and myself.

The operations were performed on July 21 at which time the tigrinum larvae ranged from 57.00 mm. to 87.50 mm. and still

possessed truly larval characteristics: their gills and fin were of enormous size, the color of the skin was still yellowish and the melanophores had not yet formed any definite pattern.

All animals were kept, before and after operation, in a cool, dark room at an average temperature of 15°C.

## 2. The results

Among the seven experiments of series XXVI only five were made in the manner described above; in some of them the eyes, in others the skin, and in still others both of these organs were examined. Abstracts from the records follow.

*Experiment V.* August 28, 1916. On skin graft network has separated into large greenish spots, which are entirely different from the light sepia spots just developing on host's skin; in this way it is very easy to identify them as Punctatum spots.

September 2. *Host on land-condition.*

September 5. On skin graft 3 yellow spots of the Punctatum type have formed definitely.

September 22. The spots on skin graft are now bright yellow.

September 25. Only 1 yellow spot of skin-graft left, which is now still brighter than before.

October 3. The grafted punctatum eye, which has not been examined until now, shows its ring broken up into yellow and black dots.

December 7. The 1 yellow spot on skin-graft has become very faint.

*Experiment III.* September 7. Yellowish spots and dots of indefinite number and appearance are developing on skin-graft.

September 22. Two grayish and 1 greenish yellow spot have assumed full distinctness and a small patch of yellowish pigment dots has formed.

September 22. *Host on land-condition.*

September 24. All spots of graft have become greenish yellow, they are distinctly to be recognized as punctatum spots and are absolutely different from the grayish sepia spots of the host.

September 29. The patch of yellow dots on the graft has developed into a single spot of the punctatum type and is extremely bright yellow.

October 4. Only 2 of the yellow spots on the rear end of the graft are still present.

October 26. One of these 2 spots has entirely disappeared, the other has been replaced by the chromatophores of the host and has turned sepia.

November 23. All spots of the graft have gone.

*Experiment VI.* Skin-graft started soon after the operation to be replaced by host's skin.

September 20. In the grafted punctatum eye the outer section of the yellow ring shows dark places.

September 23. *Host on land-condition.*

September 30. Whole ring of grafted eye broken up into yellow and black dots.

*Experiment VII.* September 25. On skin-graft, 1 yellow spot of the punctatum type has developed. Grafted eye with dark places in the outer section of the yellow ring.

September 29. The yellow spot of the skin-graft has turned brighter.

September 29. *Host on land-condition.*

October 4. On skin-graft 3 more yellow spots of the punctatum type have formed. Entire ring of grafted eye broken up into yellow and black dots.

October 10. Photographed and a few days later painted (fig. 16); compare with figure 15 of experiment IV.

October 16. On skin-graft all 4 punctatum spots still present, but 3 of them have become less dense. Only a few yellow dots are left in the grafted eye.

December 7. All 4 spots of the skin-graft have become considerably fainter, but are still present and of the punctatum type. No yellow dots left in the grafted eye.

*Experiment IV.* October 4. Host still in a larval condition. The ring of the grafted punctatum eye is still an unbroken continuous line.

October 10. Drawn and painted (fig. 15); compare with figure 16 of experiment VII.

October 26. Outer section of the ring of the grafted eye shows dark parts, inner section an unbroken yellow line.

October 27. *Host on land-condition.*

October 31. Ring of the grafted eye broken up into yellow and black dots.

November 20. Still a small number of yellow dots in lower half of iris of grafted eye present.

November 20. Photographed.

November 24. The photograph of November 20 is painted after the living animal which has not changed since that time (fig. 17).

In all five experiments the grafted organs of *Amblystoma punctatum* underwent changes typical to metamorphosis of these organs about the time that the tigrinum hosts left the water. The changes of the grafted skin were stretched, as under normal conditions, over a longer period, beginning somewhat before and ending somewhat after the host had left the water. Comparing the date of appearance of definite spots on the skin-graft with the date of the host being set on land-condition, we notice a very definite time-relation between these two phenomena: the later the latter occurs the longer the former is delayed.

	APPEARANCE OF DEFINITE SPOTS ON GRAFT	LAND CONDITION OF HOST
Experiment V.....	August 28, 1916	September 2, 1916
Experiment III.....	September 22, 1916	September 22, 1916
Experiment VII.....	September 25, 1916	September 29, 1916

Much more definite in time is the breaking up of the inner section of the yellow ring into black and yellow dots, as it never occurs before the *Tigrinum* host has left the water, a relation which is in perfect accord with the attitude of the large majority of normal *punctatum* eyes. And again, the more delayed the host's going on land, the later the breaking up of the ring occurred. In experiment 5, the eye, unfortunately was not watched when the host left the water, but one month later when the eye was examined for the first time, it showed the ring broken up into black and yellow dots. Even if we assume this to be the date of actual breaking up, this eye would have undergone the change of its yellow ring almost one month earlier than the eye of experiment 4, whose host correspondingly left the water two months later than the host of experiment 5.

	HOST SET ON LAND- CONDITION	BREAKING UP OF THE YELLOW RING OF THE GRAFTED EYE
Experiment VI .....	September 23, 1916	September 30, 1916
Experiment VII .....	September 29, 1916	October 4, 1916
Experiment IV .....	October 27, 1916	October 31, 1916

In experiment 4, the host and the graft were still in larval condition when the hosts and grafts of the other 4 experiments had metamorphosed. Since in this case the *Punctatum* graft metamorphosed far later than any *A. punctatum* larva in the experiments reported here, this experiment had particular interest. On October 10, experiment 4, was pictured (Fig. 15), and for comparison experiment 7 was pictured (Fig. 16).

Figure 15 shows the host in a perfectly larval condition, the gills and the fin of the tail still being very large. The grafted eye, which can also be seen in the picture still possesses a bright yellow ring in place of the iris; no black pigment can be detected. In figure 16, which is a painted photograph of experiment VII, the host is seen to be metamorphosed and has developed the pattern of the metamorphosed young *A. tigrinum*. The graft also is metamorphosed, as is demonstrated by its dark almost black background color and by the lemon yellow spots which it developed. The grafted eye appears very different from experi-

ment IV; it has lost its yellow ring almost entirely, only a few yellow dots remaining. The grafted eye of experiment IV did not reach this condition before the middle of November, when it was photographed and painted again (fig. 17). Only a small number of yellow dots were still present.

Thus it seems impossible to overlook in these experiments a definite time relation between the metamorphosis of the host and that of the grafted organs, which evidently means that the organs of *A. punctatum* cannot metamorphose before the host does and that they are forced into metamorphosis as soon as the host metamorphoses. The ability of one species to bring into metamorphosis the organs of another species, is the result which we wish most to emphasize among the facts disclosed by this series of experiments.

#### IV. DISCUSSION

1. The experiments reported in this paper seem to throw some light on certain fundamental principles upon which the mechanism of the metamorphosis of the Amphibian skin is based.

Let us consider first the experiments of series XXV in which from the head of one individual each of the two halves of the skin was grafted to another individual of the same species and the rate of development of the yellow pigment spots of the adult was compared in each graft with the rate of the same process in the respective host, and the other graft. Under normal conditions, i.e., if the two pieces of skin had remained with the individual to which they originally belonged, both pieces would have developed the yellow spots at the same time and would have metamorphosed simultaneously with each other. If all the factors necessary to the development of the yellow spots were in the skin itself, i.e., if this phenomenon were actually a process of self differentiation as Weigl claimed it to be, there would be no reason why two pieces of skin of the same animal should not develop simultaneously with each other even after they have been separated from this animal and transferred to different individuals.



The two pieces however, did not act in this manner. On the contrary:

a. Pieces of skin grafted from one larva to another developed the network and the yellow spots simultaneously with the host instead of with each other; i.e., they developed the characteristics of the adult skin only when the host developed them, and

b. This resulted in the establishment of a more or less considerable difference in the time of development of the adult characteristics between the two pieces of skin taken from the same animal provided such a difference existed between the two respective hosts. This difference ranged from seven days to five months with regard to network development, and from three days to four months with regard to the development of the yellow spots (separation of the network).

Such a relation can exist only if at least one factor necessary to the development of the adult characteristics of the skin is not present in the skin itself but must be furnished to the skin from the host. This factor which may be called "metamorphosis factor of the skin" is a factor external to the skin, and metamorphosis is not a process of self differentiation, for it is dependent on a body (host) which produces this factor.

It appears therefore, that in the metamorphosis of the skin a factor has manifested itself which is similar to the metamorphosis factor of the eyes and of the gills of *Salamandra maculosa*, for the metamorphosis factor of all three organs is necessary to induce metamorphosis in these organs and is primarily not contained in the organs but in the body of the organism. From experiments 33-34, of series XXV, it appears that the eye of *Amblystoma punctatum* also follows this mode of metamorphosis.

According to the method employed, the experiments described in this paper could not be expected to furnish any evidence concerning the question as to whether the second and third quality of the metamorphosis factor of the eye of *Salamandra maculosa* mentioned on the first page of this article, will be found again in the metamorphosis factor of the skin of *Amblystoma punctatum*, although this assumption seems justified. As to the fourth characteristic of this factor, we have obtained a definite answer

in the experiments of series XXVI on heteroplastic transplantation. Pieces of skin and eyes of *A. punctatum* when grafted to another species (*A. tigrinum*), showed exactly the same mode of metamorphosis as they showed when grafted to an individual of their own species. Metamorphosis of these two organs did not take place unless and until the host metamorphosed. After we have seen that neither of these two organs contain the metamorphosis factor, this can only mean that the factor has been furnished to them from the body of an individual of a foreign species. Hence the factor in question must be 'non-specific.' It was mentioned on page 239, that some evidence of non-specificity was also found for the metamorphosis factor of the eye of *Salamandra maculasa*.<sup>5</sup>

2. One objection might be raised against the method employed here. In certain cases of skin grafting the grafted pieces of skin are soon partly or entirely replaced by the host.

If, then, in the experiments of series XXV the skin graft had been replaced by the skin of the host before the appearance of the network or the yellow spots, the yellow spots developed on the place corresponding to the grafted piece of skin, would actually have been spots of the host and not of the graft. It would not be surprising then that these spots always developed at exactly the time that the rest of the spots of the host developed.

Although the fate of the grafted skin of the homoplastic series has not been followed on histological sections, there is enough definite evidence at hand to show that no replacement of the graft took place.

First: the edges of the graft retain complete definiteness throughout the larval period. This can be ascertained as the color of the graft and that of the host show as a rule at least slight individual differences (fig. 13). Later on when the animals are entirely metamorphosed, graft and host are uniformly black and the borders of the graft can no longer be distinguished. This fact does not interfere with our results, however, as at this time the phenomena on which the present investigation is based are long past.

<sup>5</sup> E. Uhlenhuth, 1913 c, p. 353.

Second: if replacement of the graft actually takes place it is easy to notice it macroscopically, without the aid of microscopic sections. This was demonstrated by the behavior of the graft in experiment 46.

Third: certain individual differences between the yellow spots of different individuals may help in determining the origin of the yellow spots on the graft, as they reappear in the graft even if the spots of the host are of a very different type from that of the graft's spots. In such cases it is easy to distinguish between the spots of the graft and the host. For instance in experiments 11-12, and experiments 21-22, there could have been no doubt that the almost orange yellow spots did not belong to the host but to the graft, even if the history of these spots were unknown. The spots of all four hosts were of a faint yellow, and orange yellow spots could not be found on their bodies except where the graft had been made. As both grafts of each pair were taken from the same individual we would expect them to develop the same type of spots, which is what actually happened. The results in experiment 33 are similar.

Fourth: Weigl,<sup>6</sup> who also studied homoplastic grafts of Amphibian skin, had experiences similar to ours concerning the possibilities of replacement of the graft by the skin of the host. According to his statements, the surrounding skin of the host never grows over or into the grafted piece of skin nor does the pigment of the graft spread into the skin of the host nor the pigment of the host migrate into the grafted skin.

The heteroplastic grafts behave very differently from the homoplastic ones insofar as they are actually replaced by the skin of the host, as will be described in another article. It will suffice to mention here that this replacement did not interfere with our experiments since it did not begin before the host had left the water (except in experiment VI). The fact that in some heteroplastic skin grafts, spots of the *Punctatum* type were formed, in itself demonstrates that the grafts were normal at least to such an extent as to produce that phenomenon upon which our conclusions are based. The same is true for the eyes

<sup>6</sup> R. Weigl, 1913, p. 597.

of series XXVI, which were grafted from the punctatum larvae to the Tigrinum larvae. They showed themselves as normal by the fact that the breaking up of the yellow ring occurred in a normal way, and as we have selected only this phenomenon for our conclusions, it is not a matter of importance what the condition of these grafted eyes was in regard to certain other histological details. Nevertheless it might be mentioned here that histological examination of the eyes proved that even several months after the breaking up of the yellow ring most of the structures and in some eyes all of the structures were still perfectly normal. Cones and rods were present and unchanged and the stroma iridis, which is the place of the phenomenon in question, showed no divergence from the normal structure of this organ. The only change observed is that the cornea is overgrown by one or more epithelial layers. The course of this process, however, can be followed macroscopically under the binocular. It did not begin before the ring was broken up and since the epithelial layers are translucent in the beginning and can be kept so for a long period by being kept wet, examination of the iris is still possible after the epithelium of the skin has commenced to grow over the cornea.

3. Concerning Weigl's opinion that metamorphosis of the skin is a process of self differentiation, we do not believe that the results of his own experiments justify such an assumption. In fact it seems that they would appear rather confusing and lacking in uniformity if metamorphosis actually were a process of independent differentiation.

Our experiments on eye grafting have demonstrated that the age of the host as well as the age of the graft—or more correctly, the stage of development of host and graft—are important considerations inasmuch as the metamorphosis factor is not carried into the eye before the host has reached a certain stage of development. If the organ be taken from an individual at this stage, it would of course develop independently from its new host. On the other hand, very young organs for some reasons respond to the action of the metamorphosis factor only after they have been grafted to the new host a certain length of time.

Hence it is clear that the true relation existing between the body of the individual and its organs cannot be studied unless attention is paid to age and developmental stage of both of them. This can only be done if the age of each individual is carefully registered and the developmental stage of each animal is recorded in definite terms, and if a special series of experiments is devoted to each of the ages and stages which play a rôle in the phenomena under discussion. Unfortunately such data are missing in Weigl's paper; it is impossible to form any conception of the age and developmental stage of such larvae which Weigl calls 'young' larvae or 'old' larvae. If the experiments are made with the point in mind mentioned above, the results become uniform and point to the conclusion that metamorphosis is not a process of self-differentiation.

Weigl's experiments may be briefly reviewed here in order to show that they do not contradict the existence of a metamorphosis factor.

First: he found that pieces of skin which were grafted from such larvae as he calls 'young,' to other 'young' larvae, always metamorphosed simultaneously with the host, which is in perfect accord with our own results, and Weigl himself expresses this fact in terms that can hardly be brought into agreement with his opinion that metamorphosis is a process of independent differentiation, since he says that the graft becomes entirely subjected to the influence of the host.

Second: he found that pieces of skin grafted from young larvae to old larvae metamorphosed some time after the host. He does not say how young the 'young' larvae were in this case nor how old the 'old' larvae were. If the 'young' larvae were only several weeks old and the 'old' larvae old enough to metamorphose a few days after the operation, this result also would be in accordance with our own results. It does not mean, however that there is any justification for the assumption that in these young grafts metamorphosis occurred independently from the host's body; it only means that the metamorphosis factor must act a certain length of time before metamorphosis in the organ can begin. If the host metamorphoses before this time is elapsed,

the grafted organ will metamorphose somewhat later than the host. Furthermore as repeatedly pointed out in this article, only one factor necessary for metamorphosis is furnished to the graft from the host, while the others must be contained or developed by the graft itself and as long as they are not—which may be the case in very young animals—the skin will not respond to the action of the metamorphosis factor. Whether we call the factors contained in the organ itself ‘structure’ as Weigl did, or whether we imagine them to be something else, does not alter the fact that the final result is brought about with the aid of the one factor contained in the body of the host, as is shown in Weigl’s experiments by the fact that the young organs after having been grafted to old larvae, metamorphosed earlier than they would have done when left with the young larvae from which they were taken. Metamorphosis therefore, manifests itself as a process of ‘dependent differentiation.’

Third: Weigl reports one experiment in which the skin of a larva which he calls ‘young’ was grafted to another larva of the same species (*Salamandra maculosa*); the skin graft metamorphosed six months before the host was photographed and the photograph shows distinctly that the host is still a larva. This case could hardly be explained on the basis of metamorphosis being induced by a factor external to the organ, for such a factor could neither have been carried into this piece of skin from the animal to which it first belonged, since this animal was a young larva according to Weigl, nor could it have been furnished to it by the host, since the host was still in larval condition. The fact that among the many grafting experiments which I had occasion to observe such a result was never obtained makes it appear doubtful whether it could be obtained at all, and there are no data even in Weigl’s paper itself which explain why he should have obtained such a result only in one case and not in other cases. We have seen that this result was obtained only when the larvae from which the graft was taken were so old as to metamorphose a few days after operation, and this seemed to be in consequence of the metamorphosis factor being already developed in such very old larvae and carried into the organ.

It is to be regretted therefore, that there are no statements in Weigl's paper as to when the individual used in the experiment in question had hatched, nor is there any picture or measurement of this individual to be found indicating the appearance of the individuals which Weigl<sup>7</sup> calls 'young.' Furthermore, although it is true that the photograph leaves no doubt as to the fact that the host was still a larva, there is unfortunately, in this photograph no definite indication that the graft had actually metamorphosed. It seems to us that the records accompanying the paper are not as complete as they should be to lead us to accept this remarkable and only exception among so many experiments.

Fourth: to a young larva of *Salamandra maculosa* a piece of skin from a young larva of the Mexican axolotl was grafted; a short time after the host had metamorphosed the skin graft metamorphosed also. It is well known that the variety of the Mexican axolotl bred in Europe, does not metamorphose and in the case before us the axolotl larvae which were kept as control did not metamorphose even after they were removed from the water and set on land. They did not show the slightest indications of metamorphosis when the grafted piece of skin metamorphosed. Weigl himself cannot but admit that the graft in this case was forced into metamorphosis by the host—even of a foreign species. In the face of this experiment, which is in accordance with the experiments reported by the writer in this article and in a former paper, and which supports the viewpoint held by the writer, it seems difficult to understand how Weigl could defend the idea that metamorphosis of the skin is a process of self differentiation.

If we compare the results mentioned in the foregoing four paragraphs we shall appreciate how much they would lack in uniformity if viewed from the standpoint that metamorphosis is a process of self differentiation; i.e., that all factors necessary to the metamorphosis of an organ are contained and developed by this organ. It seems, however, that all results, including those obtained by Weigl so far as they are based on definite

<sup>7</sup> R. Weigl, 1913, figure 7, plate XXVIII.

data, uniformly point to the one conclusion: There is in metamorphosis of an organ (i.e. in the development of the adult characteristics of an organ from the larval structure of this organ) one factor involved, which is not contained in this organ nor can it be produced by this organ.

4. In the same direction seem to point the experiments on feeding thyroid-gland to Amphibian larvae and on extirpation of the thyroid and hypophysis in amphibian larvae. These experiments demonstrate at least that the influence of certain substances which are introduced or contained primarily in the body of the animal and not in the organs in question, is in some way able to reach these organs; they demonstrate beyond any doubt that metamorphosis can be induced by an external factor not primarily contained in the organs themselves.

It seems interesting to us that a certain resemblance doubtlessly exists between the action of thyroid substance and that of the factor disclosed in the grafting experiments. First, they are both external to the organs and second, both are non-specific. Third, it seems that thyroid, like the metamorphosis factor, acts only by inducing the processes of metamorphosis; if these processes have once been induced by the action of the substance they will continue even in its absence. Kahn, for instance, stated that a single feeding with thyroid is enough to induce metamorphosis and that without any further feeding metamorphosis may be completed in a short time. We have seen that in eyes in which metamorphosis has once been induced it will be completed even if the eyes are removed from the influence of the agent and transferred to young larvae in which this agent has not yet developed. Fourth: it seems that in very young animals thyroid must act longer than in older animals in order to induce metamorphosis.<sup>8</sup> This corresponds with the fact that organs grafted from young to old larvae in which metamorphosis would occur a few days after operation, metamorphose sometime after the host instead of simultaneously with the host.

In our experiments it was not possible to demonstrate actually that the metamorphosis of all three organs examined, i.e., eyes,

<sup>8</sup> T. F. Gudernatsch, 1916, p. 358.



gills and skin, is controlled by one or more common centers, by which the factor in question is produced. The fact however, that certain important characteristics are common to the metamorphosis factor of each of these organs, encourages the belief that we are dealing in each of these organs actually with the same factor. The results shown by feeding thyroid and particularly by the extirpation of innersecretory glands seem to support this assumption.

5. We have seen that the metamorphosis of the skin of *Amblystoma punctatum* consists in the development of the yellow spots of the adult. What has been said in the foregoing pages about metamorphosis means then, that the mechanism which underlies the development of the yellow spots contains one factor which does not belong primarily to the specific properties of the skin, but is formed at some place inside of the body of the organism, at a certain time, and is carried into the skin secondarily. This factor is not specific even for the individual within the species; it is produced by every individual in the same way and therefore can be replaced for the skin of one individual by the body of any other individual of this species, without changing the final result. Furthermore: this factor is not specific even for the species as it can be produced by another species also, as the heteroplastic grafts have shown. Hence one of the most prominent characteristics of this factor is its non-specificity, it is neither specific for the organ nor for the individual nor for the species.

In addition to this non-specific factor a second factor apparently has manifested itself in the mechanism of the development of the yellow spots—it is the factor responsible for the type in which the yellow spots will develop. This factor seems to be highly specific, as suggested by the constancy with which a certain individual and even a certain piece of skin of this individual will develop a particular type of spot, and apparently lies in the skin itself.

As pointed out above, the character of the yellow spots varies greatly, and hardly two individuals can be found in which the spots are alike. The spots vary in (1) the shade of the yellow pigment, (2) the density of the yellow pigment (3) the size of

spot and (4) the number of spots. As a rule in one individual all four characteristics are developed in the same direction; thus, almost uniformly black animals are produced at one end of the scale, while very bright, almost orange yellow spotted animals are found at the other end.

If it were true that this second factor belonged to the specific properties of the skin and was specific for the individual, we should expect that a piece of skin grafted from an individual with the 'almost orange yellow' type of spots, to an 'almost black' animal would nevertheless develop on the 'almost black' host, 'almost orange yellow' spots, for the 'almost orange yellow' type of the factor should have been carried within the graft to the new host. That such is actually the case is shown by some of the experiments of series XXV. It is particularly well expressed in experiments 11-12, and 21-22. Since in each pair both grafts were taken from the same individual we should expect that if the spot developed by one graft is 'almost orange yellow,' the spot of the other graft—if this graft developed a spot at all—should be 'almost orange yellow' too; and this is what actually happened in these two pairs. A similar result was obtained in experiment 33 and experiment 45.

In a similar way must we look upon experiments 13, 28, 45, 11-12 in regard to the behavior of the hosts. In these experiments the hosts were of the black variety. This variety not only is almost bare of yellow pigment, but develops the few faint spots much later—if at all—than the majority of the individuals of this species, and at a time when metamorphosis is almost complete. In these individuals the appearance of the yellow spots cannot be said to indicate that the metamorphosis of the skin has started since the spots appear when it is almost finished, in regard to the rest of the adult characteristics of the skin. If to such an individual a normal piece of skin is grafted it will react in a normal way to the action of the metamorphosis factor and the spots will appear on it; while the host will not react in a normal way and no spots will appear, or only much later if at all, since the second factor necessary for the development of the yellow spots, being a specific factor contained in the skin itself,

is either absent from the skin of this individual, or if present is of such a specific type as to permit development of the spots only to a certain decreased amount and after a long time. This result was actually obtained in the group of experiments under discussion.

Quite similar results were furnished by the experiments of Weigl and several other investigators who studied the behavior of the transplanted skin of Amphibians.

Comparing this second factor with the first one, we see that its chief characteristic is an apparent specificity. It is specific for the organ itself, because it cannot be furnished to it by the rest of the body, and it is specific for the individual because it cannot be replaced by the corresponding factor of another individual.

It thus appears that two factors, easily distinguishable, have been revealed as involved in the process of the development of the yellow spots. One of them is a non-specific factor, the other a specific one. The first one, it seems, may be identical with a chemical substance such as a hormone; the nature of the second one is entirely unknown, but in common terms it might be called a 'specific structure.'

I should not have expounded these facts at such length if I did not think them particularly apt to throw some definite light upon the meaning of what we call 'specificity.'

From the experiments reported here, we might well form the opinion that each individual of *Amblystoma punctatum* carries in its skin a specific structure, which is predetermined and according to which the yellow spots of one individual must develop certain definite characteristics different from those of other individuals in which the corresponding predetermined specific structure was different. This structure is unchangeable as is also the type of the spots which develops from this structure; and this is what is ordinarily termed 'specificity.'

The following instance however, shows that these conclusions would have been premature. In the course of certain experiments not yet published, a series of *A. punctatum* larvae were fed on thymus gland and controlled by another series kept on

normal diet (earthworms). Both series were kept in daylight and at relatively high temperatures—up to 30°C. Many of the animals metamorphosed and developed the yellow spots. All individuals of the thymus-set, without exception showed entirely orange yellow, extremely dense spots, no matter how large or how numerous the spots were. Among the worm-fed animals there was a wider variation in regard to the color and density of the spots—from a relatively light yellow and dense type to a very intense yellow ('almost orange yellow') and very dense type; but there was not one individual whose spots were nearly like those of the thymus-animals, (i.e., nearly so reddish yellow and so dense), and this in spite of the fact that all individuals of both sets were the offsprings of one female. The difference in the color was so striking that anyone could have picked out without hesitation the Thymus animals had they been mixed up with the worm-animals. Hence there is a means to change that structure which we thought—from our grafting experiments—to be specific.

From this it seems to me that we cannot refer specificity to an individual or to a certain group of individuals, but that we must refer specificity of a certain type to the methods which we employ in trying to change the type. If our method is unfit to change it, the type appears to be specific; if our method is able to change it, the type seems non-specific. Of course, in this way, we should agree that specificity is only relative and classifying, but not absolute and not real.

#### V. SUMMARY

1. From a larva of *Amblystoma punctatum*, each of the two halves of the skin of the head, including one eye, was grafted to another larva of the same species. The animal, from which the grafts were taken, was killed; the two animals to which the pieces of skin had been grafted, constituted one experimental pair.

2. In each pair, the appearance of the color characteristics of the adult *Amblystoma punctatum* skin (network and yellow spots) in the two hosts and in the two grafts, was recorded and these dates were compared with each other.

3. The result was:

*a.* The skin grafts developed the network and the yellow spots not simultaneously with each other as they would have done if left with the animal from which they were taken, but simultaneously with the host; they metamorphosed only if the host did.

*b.* This resulted in the establishment of a more or less considerable difference in the time of development of the adult characteristics between the two pieces of skin, provided such a difference existed between the respective hosts. The difference ranged from seven days to five months with regard to the network development, and from three days to four months with regard to the development of the yellow spots. Metamorphosis of the skin graft was retarded or accelerated by the host.

*c.* The grafted eyes showed a similar time relation with regard to the breaking up of the yellow ring.

4. Pieces of skin and eyes, which were grafted from larvae of *Amblystoma punctatum* to larvae of *Amblystoma tigrinum*, not only metamorphosed also on this host of a foreign species, but showed, with regard to metamorphosis, the same time relation as on a host of the same species.

5. Individual types of the yellow spots of the skin of one individual remained unchanged even when the skin was grafted to an individual whose skin developed yellow spots of another type.

6. From these facts it is evident that at least two factors are involved in the mechanism of the development of the yellow spots of *Amblystoma punctatum*:

*a.* One factor, which is responsible for the kind of yellow spots that will develop, and which is contained in the skin itself;

*b.* Another factor which may be called 'metamorphosis factor' and which may be characterized as follows:

1. It is necessary to start the process of the development of the yellow spots; i.e. metamorphosis of the skin;

2. It is not contained in the skin; but produced by the body or some particular organ of the organism;

3. It is non-specific;

4. It may be identical with an agent like thyroid-substance.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Larva of *A. punctatum*, kept at a low temperature (about 15°C. average) in a dark room and fed on worms. It shows the first color stage (even yellow brown). Cold worms, experiment 7; hatched on May 3, 1916; land condition December 12. Photographed from live specimen October 25, natural size. (Photo 16.)

2 Larva of *A. punctatum* kept at a low temperature (about 15°C. average) in a dark room. It shows the second color stage ('Network') at an advanced stage. Cold worms, experiment 8; hatched on May 3, 1916; land condition November 14. Photographed from live specimen on October 25; natural size. (Photo 14.)

3 Larva of *A. punctatum* kept at a high temperature (about 25°C. average) in a light room and fed on thymus. It shows the beginning of the third color stage (separation of the network into yellow spots). The animal had 102 yellow spots when photographed. Warm thymus experiment 2; hatched on May 3, 1916; land condition October 19. Photographed October 9 from live specimen; natural size. (Photo 2.)

4 Larva of *A. punctatum* kept at a high temperature (about 25°C. average) in a light room and fed on worms. It shows the third color stage ('Separation of Network') in an advanced stage. The yellow spots are mostly faint and the surface of the skin appears dull. Warm worms, experiment 2; hatched May 3, 1916; land condition September 29. Photographed from live specimen October 10; natural size. (Photo 5.)





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PLATE 2.

EXPLANATION OF FIGURES

5 Eye of *A. punctatum* showing the iris divided into two sections, in a very early larval stage (about two weeks after hatching). The inner section is pure yellow and does not contain melanophores. Magnification (?).

6 Eye of *A. punctatum* about 2 months after the animal was set on land condition. The iris appears dark brown and contains only a few yellow chromatophores. Magnification (?).



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## PLATE 3

### EXPLANATION OF FIGURES

7-12 Schematic figures to illustrate size, color, density, number and distribution of the yellow spots. Printed sketches of a uniform size, showing the outlines of the animal's body, were used and the spots were drawn not in their actual size but enlarged in proportion to the size of the sketch. Spots of 'almost orange yellow' color are shown orange, while 'light yellow' spots are represented by yellow. Dotted spots are indicated by black dots on the yellow color. The borders of the graft, where they still were visible, are indicated by a white line.

7 Series XXV, experiment 21. Spot 1 is the reddish yellow spot of the graft; the spots of the host were light yellow. Drawn on December 14, 1916, from live specimen.

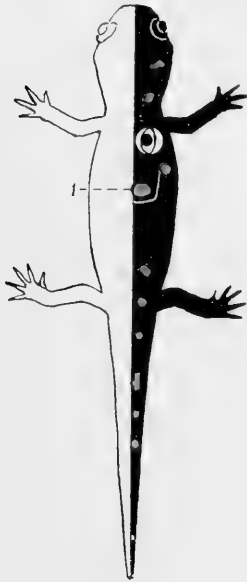
8 Series XXV, experiment 22. The spots of the host are light yellow; spots, 1, 2 and 3 are very bright reddish yellow spots. Spot 1 and 2 are spots of the graft; the history of spot 3 has not been recorded, but it probably also belongs to the graft (see text p. 258). Drawn on December 14, 1916, from live specimen.

9 Series XXV, experiment 45. The spots of the host are either pale yellow (tail) or they are faint dotted spots (body); spot 1, which belongs to the graft, is bright and almost orange yellow. Drawn December 26, 1916, from live specimen.

10 Series XXV, experiment 28. The host developed on its body only a few light yellow and mostly small spots while the largest part of the body is without spots. Drawn December 18, 1916, from live specimen.

11 Series XXV, experiment 11. The host developed only small and faint dotted yellow spots, while the graft developed a relatively large, very bright, almost orange yellow spot (spot 1). Drawn December 13, 1916, from live specimen.

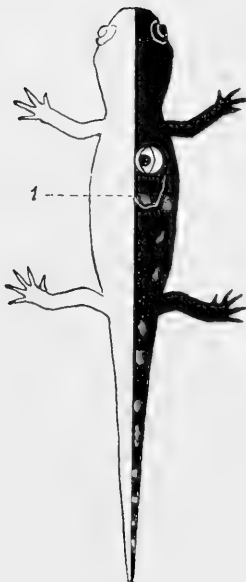
12 Series XXV, experiment 12. The host developed only 1 faint dotted yellow spot on the whole body; the graft developed 4 spots, 2 of them (spot 1 and 2) were bright and almost orange yellow. Drawn December 13, 1916, from live animal.



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## PLATE 4-5

### EXPLANATION OF FIGURES

13 A larva of *A. punctatum* with an eye graft and skin graft of a larva of the same species. The grafts were taken from the same individual as were the grafts of experiment 33 (fig. 14). Host and graft still larval; the color of the skin of host and graft is still 'even yellow brown' (without network and yellow spots); the ring of the eyes of the host and graft still an unbroken yellow line. Series XXV, experiment 34; hatched on May 5, 1916; operated on July 19; neither host nor graft had metamorphosed on January 1, 1917, when the animal died. Photographed October 19, 1916, in four times the natural size; painted a few days later from the live specimen. (When the original painting was reproduced much of the detail, particularly the distinctness of the graft's borders, was lost.)

14 *A. punctatum* with skin and eye graft taken from same animal as graft of experiment 34 (fig. 13). Host and graft metamorphosed; skin of host and graft have developed the yellow spots; the three orange yellow spots belong to the graft. The ring of all three eyes broken up into dots; only a few yellow dots still present (the picture shows these conditions only in the grafted eye). Series XXV, experiment 33; hatched on May 5, 1916; operated July 19; metamorphosed September 8; photographed October 10. Photograph enlarged four times and painted February 18, 1917, from preserved specimen and with the aid of the notes made during observation of the live animal. (The use of only a limited number of colors in reproducing this drawing has made figure 14 a mere diagram with regard to its colors.)

15 Larva of *A. tigrinum* with a skin graft and eye graft from a larva of *A. punctatum*. Host and graft still larval; host with large gills; grafted skin 'even yellow brown' (without network and spots); iris of grafted eye still an unbroken yellow line. Series XXVI, experiment IV; operated July 21, 1916; at the time of operation the host was 57.00 mm. long and the animal from which the graft was taken was 36.90 mm. long; both were larval. Host and graft metamorphosed October 27 (fig. 17). Drawn and painted October 10 from living animal; natural size. (Photo 9.)

16 *A. tigrinum* with eye graft and skin graft from *A. punctatum*. Host and graft metamorphosed. The host has no gills and has developed on the skin the colors of the young *A. tigrinum*; grafted skin has developed yellow spots of the *A. punctatum* type. Ring of grafted eye broken up into dots; only a few yellow dots left. Series XXVI, experiment 7; operated July 21, 1916; at the time of operation the host as well as the animal from which the graft was taken, were larval; size of host was not recorded; size of animal that furnished the graft was 31.20 mm. Host metamorphosed September 29; photographed October 10 (at the same time as experiment 4 in figure 15) and painted a few days later from living animal; natural size. (Photo 12.)

17 *A. tigrinum*; same animal as in figure 15, but after it had left the water. Host and graft metamorphosed. Ring of grafted eye broken up; only a few yellow dots left. Photographed November 20 (about three weeks after metamorphosis) and painted a few days later from living specimen; natural size. (Photo 13.)



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16





## THE ACTIVITIES OF CORYMORPHA<sup>1</sup>

G. H. PARKER

### 1. INTRODUCTION

In a series of papers published in the last few years I have endeavored to outline the activities of anthozoan polyps and to show the relation of such activities to the organization of these animals. The result has been a much more complex picture of the interplay of structure and function than was anticipated and a growing conviction that these polyps, contrary to the opinion of some of the most recent workers, must be admitted to contain the germ at least of much of the neuromuscular complexity of the higher animals.

Previous to my work on anthozoans I had made some study of the activities of sponges and had found in these forms a reaction system of relative simplicity. It, therefore, seemed desirable to study the activities of some intermediate type with the view of bridging over, if possible, the considerable gap between the relatively simple sponges and the much more complex anthozoans. The animals to which one would naturally turn in such a quest are the hydrozoan polyps. In most instances, however, these are of very small size and hence unfavorable for experimental work. I, therefore, sought out the largest species of hydrozoan polyp that is easily available and undertook work on it. This proved to be *Corymorpha palma* Torrey.

This species is from southern California. The material with which I worked came from the mud flats of False Bay, near La Jolla. As the work of Child and especially of Torrey has shown, *Corymorpha* is an extremely favorable animal for laboratory study, for it thrives well in an aquarium and its unusual size makes it reasonably satisfactory for experimental purposes.

<sup>1</sup> Contributions from the Zoological Laboratory of the Museum of Comparative Zoology at Harvard College. No. 300.

I, therefore, spent a large part of the summer of 1916 studying the structure and reactions of *Corymorpha*. My work was done at the Scripps Institution for Biological Research, La Jolla, and I am under obligations to the Director of the Institution, Dr. William E. Ritter, and to his staff for unfailing assistance and many courtesies.

It might seem superfluous after the very able work that Torrey ('02, '04 a, '04 b, '05, '07, '10 a, '10 b, '10 c) has done on *Corymorpha* to undertake a study of the reactions of this animal, but my objects were somewhat different from those that Torrey had in view and, though I went over much of the ground that he covered and confirmed most of what he did, I believe I have brought to light some new facts that add to our knowledge of the natural history of this most interesting species.

## 2. REACTION SYSTEMS

*Corymorpha palma* is a beautifully transparent, solitary hydroid of slender proportions. Its stalk measures as much as 10 cm. or more in length with a diameter at the thickest part of about 0.5 cm. Its proximal end is anchored in the mud and its distal end carries a hydranth that when expanded may have a spread of 2.5 cm. The proximal portion of the stalk ends in a blunt point and carries rows of long filamentous bodies, the frustules, by which it is anchored in the mud. This portion of the stalk, often half a centimeter or so in length, is commonly turned nearly at right angles to the rest, which is held vertically above the mud. A little above the level at which the stalk emerges from the mud it gains its maximum diameter; distal to this it becomes gradually more and more slender till it reaches a narrow neck, on which the hydranth is borne. The stalk itself is marked by a series of longitudinal canals inbedded in its more peripheral substance and running for the most part parallel to each other except toward the proximal end, where irregular cross fusions are common. The proximal third of the stalk is covered with a thin perisarc, the rest being naked.

A fairly well marked constriction separates the stalk from the hydranth. The hydranth consists of a thickened disc attached to the distal end of the stalk and carrying on its periphery some twenty to thirty long tentacles, the proximal tentacles. From the middle of the outer face of the disc rises the proboscis, at the distal end of which is the mouth surrounded by about forty to sixty short tentacles. Immediately beyond the bases of the proximal tentacles, and taking their origin from the proximal part of the proboscis itself, arises a number of short peduncles, to which are attached fixed medusae bearing the gonads. The appearance of *Corymorpha* as a whole is that of a delicate miniature palm whose substance seems to be translucent glass rather than animal matter.

The common reaction systems possessed by the majority of coelenterates are the mucous glands, the cilia, the nettle cells, and the muscles. In many coelenterates, as for instance the anthozoan polyps, the mucous glands are extremely important and provide abundance of secretion, whereby the column of these animals is protected and their foot and tentacles are rendered adhesive. In *Corymorpha*, on the other hand, the mucous glands are insignificant. The tentacles of this form are scarcely if at all adhesive and the other parts of its body are not especially slimy. Mucus, therefore, plays little or no part in the economy of *Corymorpha*.

It is also doubtful whether there are any cilia in this hydroid. Torrey makes no mention of these bodies in his description of the exterior of *Corymorpha* and a most careful search under the microscope with carmine suspended in seawater as an indicator has failed to reveal to me the least sign of cilia on the stalk, proboscis, proximal tentacles, peduncles of the fixed medusae, or the distal tentacles.

The longitudinal canals of the stalk when studied in a living specimen under the microscope are seen to contain a fluid carrying many minute particles of one kind or another. These particles are in almost continual motion and Torrey ('04 a, p. 417; '05, p. 334) believed that this motion was caused not only by changes of pressure due to variations in the muscular activity

of the animal but by cilia. In all the many tubes that I have examined the motion is a surging back and forth and is never local in character. I have never seen any spots in the tubes where local activity could be identified and since the surging of the fluid is fully accounted for by adjustments to muscular pressure, I suspect that there are no cilia even in the interior of *Corymorpha*. This species therefore, seems to be entirely devoid of these organoids.

Nettle cells, the third type of effector, are well developed in *Corymorpha*. Their presence can be easily demonstrated under the microscope by flooding a given piece of living tissue with dilute acetic acid, whereupon their filaments are freely discharged. They can thus be shown to be generally scattered over the stalk except that they are more abundant on the distal naked portion of this structure than on that part which is covered with perisarc (Torrey '02, p. 38; '07, p. 279). They are also found on the proboscis and the peduncles of the fixed medusae, but I have never seen them on the medusae themselves. The tentacles are abundantly supplied with them. They are especially numerous near the tips of both the proximal and the distal tentacles. In these situations two types of nettle cells can be distinguished, those with long filaments, about 0.15 mm. in length, and those with short filaments, 0.025 to 0.04 mm. in length.

The nettle cells of *Corymorpha*, like those of the anthozoan polyps (Parker, '16, p. 463), appear to be quite independent of nervous control. If a stimulating fluid, such as ten per cent acetic acid, is deeply colored and allowed to creep over a live proximal tentacle of *Corymorpha* while the tentacle is being watched under a microscope, the nettle cells will be seen to discharge their filaments only where they are covered by the stimulating fluid and never in advance of this. When such a tentacle is mechanically pressed, the discharge takes place only in the region of pressure. These strictly local responses suggest non-nervous action, a view that is confirmed by the use of anesthetics. After a tentacle of *Corymorpha* has been treated with a solution of magnesium sulphate or of chlore-

tone till it no longer responds to stimuli through its neuromuscular apparatus, it will still discharge its nettling filaments on stimulation with weak acid. It, therefore, seems clear that the nettle cells respond to direct stimulation and are not under the control of the nervous system, a conclusion that supports most of the recent work on this question in the hydrozoa (Wagner, '05, and Hadži, '09, on *Hydra*; Lipin, '11, on *Polypodium*).

The muscles of *Corymorpha* are few and simple in comparison with those of an actinian (Parker, '16) and have already been briefly described by Torrey ('04 a, p. 403). The longitudinal muscle of the stalk (Allman, '63, p. 3; '71-'72, p. 209) is a sheet of tissue whose fibers run lengthwise that structure and are closely applied to the ectodermic face of the supporting lamella.

The circular muscle of the stalk (Allman, '63, p. 4) consists of a sheet of fibers applied to the entodermic face of the supporting lamella and extending at right angles to those of the longitudinal muscle. Near the distal end of the stalk there is usually a marked constriction, which is apparently brought about by the excessive contraction of the circular muscle fibers of that region. This constriction suggests the presence of a specialized muscular sphincter, such as has been claimed by Riddle ('11, p. 390) in *Tubularia*, but as I was unable to identify in section a special accumulation of fibers in this region, it seems scarcely appropriate to describe a sphincter muscle as distinct from the rest of the circular muscle of the stalk.

An ectodermic longitudinal muscle and an entodermic circular one occur in the proboscis exactly as they do in the stalk. In both the proximal and distal tentacles longitudinal ectodermic muscles can be seen in sections. I have failed, however, to identify in the two sets of tentacles the circular entodermic muscles mentioned by Torrey ('04 a, p. 403). If they are present, they must be relatively poorly developed, for in material in which the longitudinal fibers could be clearly demonstrated, the circular fibers could not be seen though the plane of section was entirely favorable for this purpose. I have, therefore, been able to identify at most only six muscles or groups of

muscles in the polyp of *Corymorpha*: (1) the longitudinal muscle of the stalk, (2) the circular muscle of the stalk, (3) the longitudinal muscle of the proboscis, (4) the circular muscle of the proboscis, (5) the longitudinal muscles of the proximal tentacles, and (6) the longitudinal muscles of the distal tentacles. This enumeration does not include the longitudinal muscles of the peduncles of the fixed medusae nor those of the medusae themselves, whose activity, as Torrey ('07, p. 255) has pointed out, implies the presence of at least a subumbrellar band. In general the polyp of *Corymorpha* exhibits the arrangement of muscles found in the typical hydrozoan, a longitudinal ectodermic system and a circular entodermic one. The longitudinal entodermic system claimed by Lipin ('09, p. 355) for *Polypodium* is associated in that hydrozoan with a curious inversion of the germ layers that may lay this interpretation open to question.

The functions of the several muscles already enumerated for *Corymorpha* may be studied best by beginning with those of the stalk. When a fully expanded, quiescent *Corymorpha* is vigorously stimulated mechanically, its stalk shortens to about one-half its former length (Torrey, '04 a, p. 401) and at the same time thickens. Thus an expanded polyp whose stalk measured 4.7 cm. in length on contraction had a length of only 2.5 cm. In ten such instances the average contracted length was 57 per cent of the average expanded length.

If the hydranth is cut off from *Corymorpha* the contractility of the stalk remains essentially unaltered. Thus a stalk without hydranth that had an expanded length of 4.2 cm. had on full contraction a length of 2.3 cm. and in ten instances of this kind the average contracted length was 55 per cent of the average expanded length. Thus the contraction and expansion of the stalk are quite independent of the hydranth.

If the activity of the whole stalk is studied, the contraction is seen to occur in only that part which is ordinarily above the mud, the half centimeter or so of buried stalk being incapable of longitudinal contraction. This portion is devoid of longitudinal muscle fibers and hence should be expected to be non-



contractile, whereas such fibers are invariably present in that part of the stalk distal to the buried portion. The contraction of the stalk on its long axis must, therefore, be attributed to the action of its longitudinal muscle.

When the distal end of a decapitated stalk is examined, a plug of large vacuolated cells is seen to protrude from it. These cells, well known to the earlier students of *Corymorpha*, fill the axial part of the polyp and serve to a certain degree as a skeletal tissue. When the polyp contracts longitudinally, they are crowded together lengthwise and on relaxation they doubtless tend to return the stalk to its originally elongated shape. But this operation is probably much facilitated by the action of the circular muscle of the stalk, whose contraction would crowd the mass of axial cells back to its elongated form and thus restore the stalk to its original shape. Thus by the alternate action of the longitudinal muscle and the circular muscle on the mass of axial cells the contraction and elongation of the stalk is accomplished.

If live decapitated stalks of *Corymorpha* are placed in seawater containing magnesium sulphate or chloretone, they soon become incapable of longitudinal contraction. The stalks retain what would be called the elongated condition. If one of these anesthetized stalks, particularly one that has been treated with chloretone, is vigorously prodded at a given spot with a blunt rod, though no longitudinal contraction occurs, in the course of three-quarters of a minute to a minute a constricted ring appears on the stalk and remains there for from five to eight minutes. If the same experiment is tried with a stalk that has not been anesthetized, the stalk immediately contracts lengthwise but elongates soon afterwards, during which a ring of constriction appears and may remain evident for as much as ten minutes.

As the constriction rings produced in these tests must be the result of the contraction of localized parts of the circular muscle and as their formation is quite independent of anesthetization, it follows that the longitudinal and circular muscles must act upon very different principles. The longitudinal

muscle contracts much more quickly than the circular muscle does and its activity, unlike that of the circular muscle, is quite abolished by such drugs as chloretone. These features are the characteristics of a muscle under nervous control as contrasted with one brought into action by direct stimulation, and I, therefore, conclude that the longitudinal muscle of the stalk is normally under nervous influence, and that the circular muscle is stimulated directly.

Under such circumstances the interplay between the longitudinal and circular muscles in the shortening and elongation of the stalk as a result of stimulation is easily pictured. Any normal stimulus applied to the stalk gives rise to the impulses that spread through the ectodermic nerve-net and call forth an immediate contraction of the longitudinal muscle. As a result the stalk shortens nearly one-half and the axial cells become crowded together causing it to thicken proportionally. In consequence of this thickening the circular muscle is stretched, its tonus is probably increased, and it is gradually excited to action, so that on the relaxation of the longitudinal muscle the circular muscle contracts and forces the axial cells back to their original form, thus elongating the stalk. In this way the two muscles are brought into harmonious action though only one of them is under nervous control.

The longitudinal and circular muscles of the proboscis are probably organized upon much the same plan as those of the stalk, but the proboscis is too small an organ to allow of experimental tests such as have been used with the stalk. Its rapid shortening from a long, narrow organ to an almost spherical one (Torrey, '04 a, p. 397) and its slow recovery to the elongated form together with the loss of these activities under chloretone treatment all point to a condition essentially similar to that of the stalk.

In one respect only is there an observable difference between the musculature of the stalk and that of the proboscis. Occasionally the proboscis exhibits spontaneous peristaltic movements. These begin near the distal end of this organ as ring-like constrictions that progress toward its proximal end.

The time required for a single constriction to pass from one end of the proboscis to the other is from half a minute to a minute. Usually not more than one constriction is present on the proboscis at a time. The constrictions are quite obviously due to local contractions in the circular muscle. They must be very effective means of mixing the contents of the gastrovascular cavity and probably are concerned with driving some of the digestive products of the hydranth downward into the stalk. A similar movement has been known for some time in actinians and has been recently pointed out in *Metridium* (Parker '16, p. 478). Here, as there, it is probably indicative of nervous supervision of a muscle otherwise independent, but on this point no positive evidence has been obtained.

The proximal tentacles in a resting expanded *Corymorpha* radiate more or less horizontally from the basal disc to which they are attached. Each tentacle curves a little downward away from the mouth, but at its free end it turns in the opposite direction and comes eventually to point nearly outward. When stimulated mechanically such a tentacle shortens a little, perhaps a quarter of its total length, and bends vigorously inward toward the mouth (Torrey, '05, p. 333), its free end meanwhile often curling in spiral fashion through one or two turns. The tentacle then gradually relaxes and slowly returns to its original shape and position.

The axis of the tentacle is filled with vacuolated cells not unlike those occurring in the axis of the stalk and the action of the tentacle can be explained entirely on the assumption that the longitudinal muscle works against the axial cells whose elasticity returns the tentacle to its original position. In an anesthetized polyp the tentacles are always expanded in what we have assumed to be the resting position and no response can be obtained from them whatever. I, therefore, conclude on physiological as well as on anatomical grounds that there are no circular muscles in the tentacles, but that longitudinal muscles under the control of a nervous system act upon an elastic skeleton of vacuolated cells. The fact that the proximal tentacles on stimulation always bend toward the mouth would lead

one to expect that the longitudinal muscle would be more fully developed on the oral than on the aboral side of the tentacle, but of this I have been unable to find any evidence in sections of these tentacles.

The distal tentacles, which are much more restlessly active than the proximal ones (Torrey, '04 a, p. 397), in moments of rest form a cluster more or less surrounding the mouth. On stimulation they quickly jerk backward away from the mouth (Torrey, '04 a, p. 400) and point their tips toward the aboral portion of the hydranth, after which they more slowly return to their original position. On applying an anesthetic, magnesium sulphate or chloretone, to a whole hydranth or to a single isolated distal tentacle, all these reactions disappear in from one to three minutes, to reappear after the material has been for about two minutes in pure sea-water. It, therefore, seems probable that in the case of the distal tentacles the reactions are dependent upon the same interplay of parts as in the proximal ones except that the longitudinal muscles of the distal tentacles are probably more fully developed on their aboral than on their oral faces.

No attempt was made to work on the muscles of the peduncles of the medusae or on those of the medusae themselves. The peduncles contract on stimulation and the medusae are more or less rhythmically contractile. Both cease this activity when they are anesthetized with magnesium sulphate or chloretone, and both quickly recover from this condition on being placed in pure sea-water. Hence their muscles are presumably under nervous control.

Of the six sets of muscles in *Corymorpha* the two entodermic muscles, the circular muscle of the stalk and the circular muscle of the proboscis, are both slow in action and uninfluenced by such anesthetics as magnesium sulphate and chloretone. They are, therefore, probably directly stimulated and represent a primitive type of muscle such as is found in sponges (Parker '10) and in certain regions in actinians (Parker, '16). Possibly the circular muscle of the proboscis is at times somewhat under nervous control as, for instance, when that organ exhibits peri-

stalsis, but ordinarily this muscle, like that of the stalk, probably responds only to direct stimulation.

The four ectodermic muscles, the longitudinal muscles of the stalk, of the proboscis, and of the two sets of tentacles, are relatively quick in action and cease to respond under the influence of anesthetics. These are probably controlled by a nervous system composed of ectodermic sense-cells and a nerve-net, such as is commonly met with in many coelenterates (Wolff, '04; Hadži, '09).

Of the effectors of *Corymorpha* the muscles are the only elements that are under nervous control, a condition that supports the conclusion of Lipin ('11, p. 417), that in the hydroid *Polypodium* aside from the muscles no other histological elements are dominated by the nervous system.

### 3. NERVOUS TRANSMISSION

In attempting to study nervous transmission in *Corymorpha* various stimuli were first tested. *Corymorpha*, like *Tubularia* (Pearse, '06, p. 406), is apparently quite uninfluenced by light (Torrey, '02, p. 39; '04 a, p. 405). Though heat and some chemicals are stimulating (Torrey, '04 a, p. 401), these means of inducing reactions are not so easily controlled as mechanical stimuli (Torrey, '02, p. 41). But even here a tremor may be transmitted from the part of the body touched to a distant receptor and thus call forth deceptive responses. I, therefore, resorted for stimulation to a weak faradic current; the electrodes for this could be set in place and, after all possibility of accidental mechanical disturbance had passed, the intended stimulus could be applied by simply making the current, thus avoiding any complication from possible tremors. A well localized and controllable stimulus was thus obtained.

If a proximal tentacle of *Corymorpha* is gently touched by a blunt glass-rod or stimulated by a very weak faradic current, it will in a second or so after the application of the stimulus bend rather quickly toward the proboscis, after which it will slowly return to its resting position. A more vigorous stimulus will excite to action not only one but many of the proximal

tentacles (Torrey, '04 a, p. 399); the proboscis is also very likely to turn in the direction of the stimulated tentacle, and the distal tentacles may likewise become active. To a still more vigorous stimulus not only do the parts of the hydranth respond, but the stalk may contract (Torrey, '04 a, p. 400). Thus impulses to motion may spread from a single proximal tentacle to any part of the body. The same is true of the proboscis and of the distal tentacles. Conversely, by stimulating the stalk, responses can be called forth from both sets of tentacles (Torrey, '04 a, p. 401). These observations indicate a diffuse type of transmission, such as is generally assumed for a nerve-net. It is also clear that the stronger the stimulus the more distant the effector that can be activated.

To ascertain something of the nature of the transmission, several kinds of experiments were tried. When a polyp of *Corymorpha* is anesthetized for a few minutes with magnesium sulphate or chloretone, all responses of tentacles, of proboscis, and of stalk disappear except the slow formation of constriction rings in the stalk. The responses thus eliminated may be made to return by placing the polyp for three or four minutes in pure sea-water. Since the effectors that are rendered inactive are the ectodermic musculature and since the drugs used are known to act chiefly on nervous tissue, the conclusion is drawn that the essential part of the nervous mechanism of these animals must be in their outer layer, the ectoderm, a conclusion supported by the very short time needed for anesthesia as well as for recovery from this state. It, therefore, seems probable that nervous transmission in *Corymorpha* is an affair of the ectoderm.

To test this view, a polyp about ten centimeters in length was put horizontally in an aquarium and pinned at the middle of its length to a slight elevation of wax so that its foot-end and its hydranth projected for some distance horizontally and freely into the surrounding water. On applying a faradic current to the hydranth, the foot-end contracted together with the rest of the stalk; on applying it to the foot-end the hydranth contracted together with the rest of the stalk. The polyp was now un-

pinned, bent into the shape of a U with the curve where the pin had been and this part was dipped for from one to two seconds into ten per cent acetic acid and then immediately immersed in pure sea-water. As a result of this treatment the ectoderm was killed in a broad ring around the middle of the stalk, but the deeper tissues, at least the axial vacuolated cells, were left alive. On pinning the polyp again in the horizontal position and stimulating it electrically, it was found that the nervous impulse never passed from one end to the other, as in the first trials, but was always limited to the end at which it was applied, showing that the superficial tissue of the polyp, but not its core, is the part concerned with transmission.

This experiment was repeated with the modification that, instead of destroying the superficial tissue with acetic acid, it was anesthetized with chloretone. Polyps thus treated failed to transmit impulses over the anesthetized region though after ten minutes in pure sea-water transmission in this region was reëstablished. As chloretone is known to abolish the neuromuscular activities of the ectoderm, but to leave the entodermic muscles unaffected, I conclude that the parts concerned with transmission are not only superficial but ectodermic.

To determine whether there are special transmission tracts in the polyp, several lines of experimentation were carried out. A large polyp was split lengthwise from the hydranth end through the column almost to the aboral end. Such a preparation has the shape of a letter V, with two half-hydranths at the free ends of the V and the unsevered base at the angle. On stimulating one half-hydranth with a faradic current both arms of the preparation contracted though the base was outwardly inactive. Hence I conclude that the base, though without muscle, transmits nervous impulses from one side of the polyp to the other. If the base is now locally anesthetized and the experiment repeated, the contraction is found to be limited to the arm of the preparation that is stimulated, thus demonstrating the superficial location of the transmitting tissue.

I next tried another form of this experiment in which, however, the two arms of the preparation were united through the

hydranth instead of through the base. In this experiment transverse transmission through the hydranth was as easily demonstrated as it had been through the base.

In a third experiment the stalk alone was used, the hydranth and base having been cut off. When one arm of such a preparation was stimulated the other arm almost invariably failed to respond thus showing that though transverse transmission is easily accomplished in the base or in the hydranth, it is not so easily accomplished in the stalk, where longitudinal transmission is the rule.

The predominance in the stalk of longitudinal as compared with diffuse transmission makes localization a significant feature in the responses of this part. If a faradic stimulus is applied to one side of the stalk next the hydranth or next the base, the stalk simply shortens as a whole. If, however, the stimulus is applied on one side of the stalk midway its length the stalk bends to that side and usually presses the hydranth with great accuracy against the stimulated spot. This response is not only appropriate for the particular side stimulated but also in most cases for the given level of the stimulated point on that side. The significance of these responses to localized stimulation was often observed in the stock aquarium. This contained by accident a number of small nudibranch gastropods, which were found to feed on the substance of *Corymorpha*. When one attacked a *Corymorpha*, it began at the base of the stalk where the hydroid rose from the mud and as soon as it started to nibble the stalk on a given side the *Corymorpha* responded by applying to the point of attack the hydranth, the tentacles of which were extremely stimulating to the nudibranch and usually drove off the intruder. The success of this form of protective response naturally depended upon the accuracy of the localization.

To ascertain whether transverse transmission is at all significant in the stalk of *Corymorpha*, the following experiment was tried. The stalk of a polyp was cut transversely halfway through at a point midway its length and the polyp was then allowed to come to rest in a vertical position. On stimula-



ting locally below the wound and on the side away from it, the hydranth, as might have been expected, was applied accurately to the stimulated spot. On applying the stimulus directly below the wound the hydranth was turned to that side but never descended far enough to cover the actual region of the stimulus. The failure here seemed to be due to the deficiency in the musculature as a result of the operation rather than a defect in transmission. Since a decapitated stalk responds to local stimulation in the type of experiment just described with as much success as a normal one does, I conclude that, though longitudinal transmission is the predominate feature of the stalk, transverse transmission also occurs in this part of *Corymorpha*.

This conclusion is supported by the observation that a decapitated stalk which has been partly cut through transversely at several different levels and from several different sides, as Torrey ('04 a, p. 407) has already described, will nevertheless localize, though incompletely, a stimulated point.

From the observations and experimental results recorded in this section, it seems fair to conclude that nervous transmission in *Corymorpha* is very probably limited to the ectoderm and is diffuse, except that in the stalk longitudinal transmission predominates much over transverse. Notwithstanding this primitive state of nervous development, reactions that have all the essentials of a nervous reflex may occur. Thus, when a proximal tentacle is vigorously stimulated, not only does it and some of the adjacent tentacles respond, but the proboscis commonly turns toward the point of stimulation. This accurate form of response of a distantly located organ to a circumscribed stimulus has all the characteristics of a reflex, though it is probably dependent upon the activities of a nerve-net which has the capacity of calling into action more easily those muscles that lie near the receptive position than those that lie far from it.

## 4. INDEPENDENCE OF PARTS

Enough has already been said to make it quite clear that many of the parts of *Corymorpha* are quite independent of the rest of the polyp in their capacity to respond to stimuli. Thus the stalk of *Corymorpha* will shorten and even localize a stimulus applied to one side of it with as much success after the hydranth has been removed from it as when the polyp is completely intact. There is, therefore, no reason to suppose that the hydranth contains nervous centers that are in any way essential to these responses.

In a similar way the separated hydranth exhibits on stimulation movements in its distal and proximal tentacles and its proboscis that are in all respects counterparts of the movements of these organs in the polyps as a whole, showing that the hydranth is in no sense dependent upon the stalk for its neuromuscular activity. The separate hydranth as well as the separate stalk can be anesthetized with chloretone or magnesium sulphate and, after having thus lost their responsiveness, these parts will separately recover when placed in pure sea-water.

When the proboscis of a *Corymorpha* is cut off, the attached distal tentacles continue to exhibit spontaneous movements and turn vigorously away from the mouth on stimulation as they do on the intact animal, activities that they lose temporarily on anesthetization. The proximal tentacles also exhibit great independence. If one is cut from a hydranth, its curved form brings it to rest on the bottom of a glass vessel on its side and it is comparatively easy to determine by the direction of its curve which is its oral and which its aboral face. On stimulating it mechanically, it shortens slightly and curls orally as an attached tentacle does. The same is true when it is stimulated by a faradic current. Under this more favorable method of stimulation a proximal tentacle that had a length of twelve millimeters when at rest shortened to ten on stimulation and curled to a half circle. These reactions disappear after the isolated tentacle has been for five minutes in sea-water containing chloretone and reappear on transferring it for three minutes to pure sea-water.

If an isolated proximal tentacle is cut in two near the middle, the distal piece coils and the proximal piece curves in response to a faradic stimulus as when they were parts of the whole tentacle. Both parts, moreover, continue to show slight spontaneous movements as the normal tentacle does. All these responses disappear on anesthetization and reappear after the fragments of tentacle have been for a few minutes in pure sea-water.

It is quite evident from these observations that the neuromuscular organization of *Corymorpha* is most diffuse and contains nothing that can rightly be looked upon as centralized. In this respect the hydrozoan polyp is, if possible, more a congregation of parts than the anthozoan polyp, which, as I have elsewhere attempted to show (Parker, '17 b), lacks very largely that centralization feature that is so characteristic of the neuromuscular structure and activities of the higher animals.

#### 5. LOCOMOTION

Although *Corymorpha* is usually described as a fixed polyp, it possesses, as Torrey ('04 a, p. 416) has shown, some slight powers of locomotion. When the buried end of a *Corymorpha* normally affixed in the mud is carefully examined, it is found to be more or less ensheathed in a tube of its own secretion. If such an animal is freed from the surrounding sand and mud and is placed on a small glass plate, it soon attaches itself by the secretion of a new tube and by its frustules and moves slowly along producing new tube material as it goes. The highest rate of locomotion that I have ever observed is seven millimeters in twenty-four hours, though Torrey ('04 a, p. 416; 1907, p. 278) has recorded about twice that amount in the same length of time. In any event the rate is relatively low and the animal is essentially fixed. I have made no observations on the method of locomotion. The rate favors Torrey's view ('04 a, p. 415) that this process is accomplished by the amoeboid activity of the basal ectoderm, a procedure that is claimed to occur in *Hydra* and is probably employed by the larva of *Corymorpha* as it creeps out of its egg capsule (Torrey, '07, p. 259).

## 6. GEOTROPISM

*Corymorpha palma* when seen under natural conditions submerged in water on the mud-flats is almost always found standing upright. Its buried end is as a rule turned at a considerable angle to the chief axis of its stalk, which when uninfluenced by tidal currents and the like is almost invariably vertical. The constancy of this position can be tested by the following means. An iron nut the hole of which has been filled with paraffine can be used as a convenient base to which to attach a *Corymorpha* by pinning the mass of tangled filaments, sand, and the like at the end of its secreted tube. An animal once thus firmly attached can be conveniently put in almost any position. If the nut is placed on the bottom of an aquarium so that the stalk of the *Corymorpha* is vertical, the polyp will remain indefinitely in that position. If the nut is turned over on its side so that the stalk of the polyp is horizontal, in an hour or two the polyp will be found to have made a sharp bend whereby it has regained its vertical position.

If a polyp is allowed in the course of a day to attach itself firmly to a small glass plate, it will be found to rise vertically from the plate. If the plate is now held on edge the polyp soon changes its direction and brings its axis to lie parallel to the plane of the plate with the hydranth uppermost. If the plate is turned upside down and suspended in the aquarium in this position thus causing the polyp to head vertically downward, in a very short time the polyp will turn to the horizontal and rest there against the underside of the plate. Thus with every turn the polyp endeavors to get as near to a vertical position with its hydranth above as is possible. Negative geotropism is, therefore, a well marked feature of *Corymorpha*.

That the position assumed by *Corymorpha* is not the result of some such purely mechanical condition as the specific gravity of its parts has already been shown by Torrey ('04 a, p. 404), who demonstrated that the whole polyp is heavier than seawater and that the hydranth has a higher specific gravity than the stalk.

I have confirmed these observations and have extended them by making measurements of the specific gravity of the several parts. If a strong solution of ordinary cane-sugar in sea-water is added to sea-water in which a clean *Corymorpha* is floating, it is comparatively easy to arrive at that concentration at which the *Corymorpha* is just suspended. The specific gravity of such a solution can then be determined by a hydrometer and thus that of the *Corymorpha* ascertained. By this method I found that the specific gravity of an expanded *Corymorpha* was 1.0292, of a contracted one, due probably to the loss of water, was 1.0320, of the stalk was 1.0268 and of the hydranth was 1.0530. The sea-water in which these animals were living had a specific gravity of 1.0250. It is clear from these records that the negative geotropism of *Corymorpha* is in no sense the direct result of the specific gravity of the whole animal, of the stem, or of the hydranth. It must, therefore, be an indirect response of some kind.

If a *Corymorpha* is attached to a movable base, its hydranth cut off, and the base so set that the stalk is in a horizontal position, the stalk soon turns and assumes a vertical posture. The rates of turning are about the same in the headless polyp and in the whole one. Thus in one set of experiments five stalks that had turned from horizontal to vertical in the average time of one hour and forty minutes made the same turn after the loss of their hydranths in one hour and fifteen minutes. In a second set the normal individuals became vertical in an average period of one hour and ten minutes and after the loss of hydranths in one hour and thirty minutes. Thus there seems to be very little difference between the geotropic response of a normal polyp and of a stalk without a hydranth. The geotropic response must, therefore, be a feature of the stalk.

If anchored stalks are placed horizontally in sea-water containing enough sugar in solution to make it of the same specific gravity as the stalk, the stalk soon takes on a vertical posture. Evidently the stimulus to geotropism is not due to the weight of the stalk pressing upon its support but must depend upon

some internal mechanism such as small heavy particles contained within its substance.

The means by which the geotropic response is accomplished is in no sense clear. Torrey ('02, p. 39; '04 a, p. 403; '05, p. 334; '10 b) in a series of experiments was led to favor the view that the response was the result of tension or growth changes in the vacuolated axial endoderm and not due to neuromuscular activity, thus bringing the reaction in line with the methods known to obtain in plants rather than in animals. This view was supported by the slowness of the reaction, a feature common in the geotropism of plants, and by the fact that if numerous transverse cuts are made in the stalk of *Corymorpha* so as to interrupt the longitudinal muscle but leave the axial cells essentially intact, the response is still carried out though imperfectly.

I have confirmed Torrey's observation that a stalk of *Corymorpha* cut as he described will still exhibit negative geotropism, but I have also shown that transverse transmission occurs in the *Corymorpha* stalk and that, therefore, neuromuscular activity may spread around cuts such as Torrey made; it is, therefore, still possible that this response may depend upon the muscles rather than the contained parenchymatous cells. To test this question I employed other methods than those used by Torrey.

The parenchymatous core cells in *Corymorpha* can be very generally destroyed by passing a rough needle down the axis of a stalk and then gently twirling it round and round. As a result of this treatment the stalk is left as a rather limp tube but one which nevertheless in seawater has some resistance. Six such stalks with their axial cells bored out were mounted in horizontal positions and their responses watched. Five of these eventually turned up into approximately vertical positions in times that ranged from two hours to four hours and a half. One never moved much out of the horizontal. An examination under the microscope of the interior of those that responded showed that their axes were filled with a pulp of vacuolated cells and cell fragments that made it clear that these cells played

no essential part in geotropism. The motionless one was found to have the ectoderm layer much torn.

I next attempted the converse of the preceding experiment and tried to destroy the neuromuscular mechanism and leave the axial cells unharmed. Stalks that were known to possess well marked negative geotropism were treated for ten seconds with two per cent acetic acid to kill the ectoderm and were then placed horizontally in an aquarium. In the course of five hours no geotropic response had appeared in these stalks and the experiment was concluded by a microscopical examination of them. From each stalk a skin could be peeled off leaving an axis of vacuolated cells whose consistency was that of a fairly stiff jelly. In histological appearance these cells seemed to be entirely normal and precisely like those that might be taken from a freshly dissected living polyp. 'I, therefore, believe that the absence of geotropic reactions in these animals is not due to any defect in the axial cells but is the result of the loss of the neuromuscular coat.

Another way of testing this matter was by the use of anesthetics such as chloretone. Stalks of *Corymorpha* were placed in a vertical position in sea-water containing chloretone till their neuromuscular responses ceased whereupon they were turned sidewise so that their chief axes were horizontal. All remained in this position for five hours after which they were put in pure sea-water. Within an hour after this change had been made they began to show geotropic movements. Here too the evidence favors the view that the negative geotropism of *Corymorpha* is dependent upon neuromuscular activity.

Although I do not believe that the axial cells of *Corymorpha* take any active share in the geotropic responses of this animal, I am convinced that they play a considerable, though passive, part in this response. If the headless stalk of a *Corymorpha* is inserted into a horizontal glass tube so that only the distal half of it projects from the end of the tube into the surrounding water, the stalk will bend upward where it is free and assume in a short time a form not unlike a letter L. If after having been two hours in this position, it is released, it

will retain this bent form for at least twenty minutes. I, therefore, believe that the axial cells have the character of a plastic skeleton, as in fact Torrey ('07, p. 201) has stated, and that they will take a certain amount of set, from which they are not quickly and easily freed. This set may be due to the molding effect of the musculature or to changes in their turgescence or to both. In this respect they seem to me to be important adjuncts in the geotropic responses of *Corymorpha*. These responses, however, are accomplished primarily by the neuromuscular apparatus (Parker, '17 a), as has been shown by Loeb ('95) for *Cerianthus*.

#### 7. FEEDING

If the contents of the digestive spaces in *Corymorpha* are examined, they are found to contain, as Torrey ('04 a, p.397) has observed, organic detritus composed of the remains of copepods, rotifers, diatoms, and various chlorophyl-bearing protista. The cavity in which these partly digested materials circulate is by no means simple. Its extent and ramifications can best be made out by the injection into it of india ink under slight pressure. In this way it can be demonstrated that the body of the hydranth including the proboscis contains a considerable gastrovascular cavity (May, '03, p. 585). Extensions of this reach out into the peduncles carrying the medusae but not, so far as I can ascertain, into the tentacles. Proximally the gastrovascular space contracts into a central tube as it passes from the hydranth into the stalk, at the distal neck of which it connects with the dozen or more longitudinal canals which extend in some what parallel courses proximally through the more peripheral layer of the stalk well down to its buried end. These tubes show not infrequent anastomoses, especially in the proximal portions of their course. The injection of the whole gastrovascular system is best made by cutting off the proximal tip of a polyp, inserting a cannula in among the vacuolated cells, and after tying it in that position, slowly injecting india ink under slight pressure. The ink quickly makes its way into the canals and flows forward freely till the neck between the stalk



and the hydranth is reached. Here commonly a slight block is encountered, due probably in part to the contraction of the sphincter in the circular muscle of the stalk and in part, perhaps, to the fenestrated membrane (Torrey, '07, p. 283). By continued slight pressure the ink will suddenly spurt through this neck and quickly fill the cavity of the hydranth where again it may meet a block if the mouth is closed. This obstruction, which seems also due to a sphincter, may likewise be overcome, whereupon the ink will flow in a fine stream through the mouth into the outer water. I have never been able to discover any evidence in favor of the view expressed by some of the older workers that extensions of the gastrovascular cavity make their way from the canals into the regions between the vacuolated cells of the stalk (Agassiz, '62, p. 278) or from the cavity in the hydranth to the axes of the tentacles (Allman, '71-72, pl. 19, fig. 7).

The gathering of food into the gastrovascular cavity is one of the best organized and most characteristic sets of responses in *Corymorpha*. In an aquarium through which a gentle current of water has been sweeping and in which the specimens of *Corymorpha* have become well acclimated, they will be found well fixed in the silt, with stalks upright and with their hydranths pointing in the direction in which the current flows. If the current is now shut off, feeding movements will begin in about three to four minutes. These have been briefly described by Torrey ('04 a, p. 397) and consist of the following steps. At the beginning of a feeding movement the expanded proximal tentacles with two or three convulsive efforts are drawn together around the proboscis and at the same time the stalk is shortened. The stalk then curves so that the hydranth is brought close to the mud. The proximal tentacles are opened and the distal tentacles and proboscis are applied to the mud. The stalk then slowly straightens and becomes vertical, the proximal tentacles attaining full expansion as the resting position from which the response started is again assumed. The whole response partakes of the nature of a deep bow and requires for its completion about one minute. As a result of it many small

bits of detritus and the like become entangled among the distal tentacles, whence they are transferred to the mouth. The proximal tentacles seem to play a very minor part in this type of food gathering. Records on a single individual showed that in one hour twenty feeding movements were made. Of the three minutes involved in each interval, for the intervals were very regular, about one minute was taken up in the actual feeding movement and about two in rest.

When a *Corymorpha* that has been exhibiting a regular sequence of feeding movements in quiet water is subjected to a current of water, these movements cease at once, to begin again a few minutes after the current has been cut off. Apparently this type of response in normal animals is dependent upon quiet water.

If a *Corymorpha* that is feeding regularly in quiet water, is deprived of its hydranth by having it suddenly clipped off at the neck by scissors, the stalk continues the feeding responses but without bowing low enough to bring its distal end close to the substrate. In this sense the response is defective and, as the following record shows, it is also slower than before. A normal polyp was found to carry out feeding responses at the following intervals in minutes, 3.5, 3.5, 2.5, 2.5; whereupon its hydranth was cut off and the stalk continued to respond at intervals in minutes of 5, 4, 5, 4.5, and 5, when the experiment was concluded.

Another curious effect of beheading a polyp was seen in experiments with pairs of feeding individuals. A pair of polyps regularly feeding in quiet water were watched for a short time and then the hydranth of one was cut off; both individuals the one without a hydranth as well as the one with a hydranth continued to carry out bowing movements. The circulating current in the aquarium was then turned on whereat the normal individual ceased to respond while the headless one continued to carry out rhythmic bowing movements. This experiment was often repeated and with uniform results. The water currents in some way stimulate the whole animal so as to cause the feeding responses to cease, a condition that does not obtain in the

case of the stalk alone. This response gives more evidence of nervous integration in *Corymorpha* than any other with which I am acquainted.

After the separation of the hydranth from the stalk, not only does the stalk continue to respond but the distal tentacles of the hydranth close and open at regular intervals as they did in the normal feeding responses; the rates of response in the two parts are, however, quite different. Thus in a normal polyp whose feeding movements had been carried out for some time at intervals of 2.5 to 3 minutes the hydranth and stalk were separated, after which the stalk responded every 8 to 9 minutes and the hydranth every 3.5 to 4 minutes. Thus each part individually had a slower rate than the whole animal, the stalk being much slower than the hydranth. It would be natural to expect that one or other of these parts might serve as a pace-maker for the whole system, but of this there is no evidence.

In a similar way a stalk may be cut crosswise in halves and the two halves will continue to show rhythmic contractions. As in the former case, both halves have a lower rate than the whole stalk had. Possibly in both cases the reduced rates give evidence of a general control which is somewhat disturbed by the cutting, though of this there is no conclusive evidence.

Besides the type of feeding that has just been considered and that is apparently characteristic of quiet water, a second type is also to be noted (Torrey, '04 a, p. 397). When detritus of one kind or another is carried by a gentle current on to the expanded proximal tentacles of an erect *Corymorpha*, these are very likely to wave inward carrying everything with them toward the distal tentacles, which in turn move quickly outward to meet the incoming proximal members and eventually transport their booty to the mouth (Torrey, '04 a, p. 402.) In this way under favorable circumstances much food is doubtless obtained, but the success of this operation is much more dependent upon accident and the whole procedure seems to have less organized effort about it than the plan of feeding described for quiet water.

The food accumulated by the two methods mentioned in the preceding paragraphs doubtless undergoes digestion in the gastrovascular cavity of the polyp and is moved about in this cavity by the peristalsis of the proboscis already referred to.

#### S. GENERAL CONCLUSIONS

When the responses and activities of *Corymorpha* are compared with those of an anthozoan polyp, their inefficiency is most striking. This is especially well seen in the tentacular responses to food. In an anthozoan the tentacles when touched by a piece of food turn in many directions till they have more or less entwined the food. They become covered with a sticky mucus and they discharge their nettling filament with great freedom. Finally by the action of their cilia and muscles the food is delivered at the lips. In *Corymorpha* the proximal tentacles are not provided with mucus and their one muscular response is to wave toward the mouth, a response that occurs as well when the food touches their aboral faces, and is consequently left behind by their response, as when it is on their oral face. No cilia are present in *Corymorpha* to help transport the food to the mouth. In *Corymorpha* the whole process of food gathering has a strongly marked mechanical character that makes it much less successful as a means of getting all the food within reach than the operations carried out by the anthozoan tentacle (Torrey, '04 a, p. 401). This lack of close adjustment, that has been noticed in the tentacles of *Tubularia* (Pearse, '06, p. 403) as well as in those of *Corymorpha*, runs through all the reactions of *Corymorpha* as compared with those of the anthozoan polyps.

Notwithstanding the general inefficiency of the responses of *Corymorpha*, this polyp contains among its muscles about the same array of types as are found in the actinians. Some muscles, like the circular muscle of the stalk, are apparently quite without nervous connections and respond to direct stimulation; others, like the circular muscle of the proboscis, probably usually respond to direct stimulation though they may be influenced by nervous impulses; and finally muscles, like the longitudinal

muscle of the stalk, are completely under nervous domination. Some of these, such as the longitudinal muscle of the proboscis, exhibit responses which are called forth in such a way that they are indistinguishable from a reflex in the higher animals. From this standpoint *Corymorpha* reproduces in miniature all the conditions found in anthozoans. And this is further emphasized by the lack of any general nervous center and the consequent great independence of all organs from the side of their neuromuscular activity. *Corymorpha*, therefore, does not seem to fill the gap between the extremely simple effector system of sponges and the receptor-effector systems of anthozoans, but rather presents a reduced though not simplified state of the anthozoan type. If the muscles of *Corymorpha* were more commonly open to direct stimulation than they are and if its activities presented less that can be interpreted in the nature of a reflex, it might supply more nearly the requisites of an intermediate type, but as it is it resembles rather a reduced anthozoan than a form in any sense intermediate between sponges and sea-anemones.

#### 9. SUMMARY

1. Of the four classes of effectors commonly found in coelenterates, mucous glands, cilia, nettle cells, and muscles, *Corymorpha* possesses only the last two. The nettle cells are not under the control of the nervous system; the muscles may or may not be so controlled.

2. *Corymorpha* has four ectodermic muscles: the longitudinal muscle of the stalk, the longitudinal muscle of the proboscis, and the longitudinal muscles of the proximal and of the distal tentacles. These four muscles are under the control of the nervous system. It has two entodermic muscles: the circular muscle of the stalk and the circular muscle of the proboscis. Both are stimulated directly, but the second is probably also under partial nervous control,

3. Nervous transmission in *Corymorpha* is diffuse except in the stalk, where it is predominantly longitudinal.

4. The stalk, proboscis, and tentacles in *Corymorpha* exhibit great autonomy, in that each one possesses its own neuromuscular mechanism.

5. Locomotion is extremely circumscribed and slow.

6. *Corymorpha* is negatively geotropic and this response is neuromuscular in its origin.

7. In quiet water *Corymorpha* feeds by inverting the hydranth and pressing the mouth and distal tentacles on the mud. In running water the proximal tentacles entangle food and transfer it to the distal tentacles and the mouth.

8. The neuromuscular mechanism of *Corymorpha* is not intermediate between that of the receptor-effector system of actinians and the independent effectors of sponges. It resembles a reduced actinian system rather than a primitive state from which such a system could be derived.

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THE COMPARATIVE PHYSIOLOGY OF SYNAPTULA  
HYDRIFORMIS (LESUEUR)<sup>1</sup>

J. M. D. OLMSTED

TWO FIGURES

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INTRODUCTION

Two varieties of *Synaptula hydriformis* (Lesueur), red and green, are found at Bermuda in approximately equal numbers. All gradations between these two colors are present, and there are

<sup>1</sup>Contributions from the Bermuda Biological Station for Research. No. 74

many quite transparent individuals of a very pale flesh color which appear light green when placed on green sea-weed, and a light red on red sea-weed. Clark ('07) reports that he found synaptulas more abundant on green Ulvaceae, but I collected comparatively few specimens from green sea-weed, obtaining them almost exclusively from red algae growing in shallow water on broad sheltered flats (Millbrook Creek). There was absolutely no 'outspoken mimicry' of the environment by the animal such as Semon ('87, p. 280) describes for several species, since both red and green synaptulas occur on both red sea-weeds and green ones. Dr. W. J. Crozier informs me that he has found this species in August on the outer reefs in the brown Sargassum, and that the red and green varieties were present there in equal numbers. In my collecting, by far the greater number were taken at the base of clumps of red sea-weed, where a slate color or even black prevailed (Clark, '98, p. 56).

The work was done at the Bermuda Biological Station for Research during the summer of 1916. I wish to thank the trustees of the Humboldt Fund and Dr. E. L. Mark for making it possible for me to go to Bermuda, and also Dr. W. J. Crozier for suggesting this species to work upon. Further acknowledgment is due Dr. Mark for his helpful criticism of the manuscript.

## I. GENERAL PHYSIOLOGY

### 1. *Locomotion*

Locomotion through sea-weed is accomplished largely by means of the tentacles, though movements of the body in the form of peristaltic waves assist. That the tentacles subserve locomotion, as well as the sense of touch, in holothurians was first shown by Tiedemann (1816). The locomotor function is so emphasized in this particular species that Lesueur ('24) in his original description of the species says: "In locomotion the tentacles are used as feet." It is interesting to note that the second time this species is mentioned, this characteristic is again referred to, for "there can be no doubt that Pourtalés' ('51) *Synapta* from Biscayne Bay, which he called *viridis*, is identical

with Lesueur's and Oersted's West Indian species" (Clark, '07, p. 83). Pourtalés reports that when specimens of this species were placed in a glass jar, they would climb along the sides of the vessel by means of their tentacles, their bodies hanging down in the water, and they seemed to adhere to the glass by the outside surface of their tentacles.

The body movements are much like those of the earthworm. Indeed, the arrangement of the muscles is the same in both animals; circular muscles lie just beneath the epidermis, and longitudinal muscles internal to the circular ones. At one phase of the movement the circular muscles contract and the body of the *Synaptula* is elongated. This is succeeded by a contraction of the longitudinal muscles—following von Uexküll's ('00) rule whereby the stretched muscle is the next to contract,—which shortens the body. Instead of setae, *Synaptula* has calcareous anchors, which serve as fulcrum when the animal is moving, or as very efficient hold-fasts when one attempts to remove a specimen from sea-weed. Concerning the function of these peculiar structures, which have their origin in the dermis, Selenka ('67) ventured to suggest that they grew out beyond the subcuticula, bored through the thin membranous outer skin, and aided the *Synaptula* in clinging to the substrate. Semper ('68) on the contrary, claimed that "ein solches Durchboren findet aber nie in natürlichen Zuständen statt," and he suggested a totally different function, i.e., that of 'Tastorgane.' Nevertheless the anchors do project beyond the epidermis and render it almost impossible to remove a *Synaptula* from sea-weed without injury. In collecting specimens, it was found best to break off a bit of sea-weed with an animal clinging to it, rather than to attempt to pull the animal away from the sea-weed. They even cling to one's finger so that it is with difficulty that they can be shaken off.

Naturally anchors are of no avail when the animal is creeping up the vertical surface of a smooth glass vessel. In this case the whole outer glandular portion of the tentacles (cf. Pourtalés, '51), not merely the tips as in *Rhabdomolgus ruber* (Becher, '07, p. 554), adheres to the surface along which the *Synaptula* is moving. The longitudinal muscles of the tentacles contract,

thus pulling the body forwards, and immediately another tentacle is attached above the first. There is no twisting of the tentacles such as Clark ('99) describes for *Synapta inhaerens* and *S. roseola*. The adhesion to the glass is so strong that a tentacle presents the appearance of being almost torn away when it finally lets go. The effectiveness of the pull of any tentacle ceases when another tentacle, attached higher than the first, begins in its turn to contract and pull the body up, yet an attached tentacle seldom loosens its hold until it has assumed an 's' shape, the proximal end having reached the same level as the distal end. This is what one would expect if the tentacles were attached to the glass merely by some adhesive substance and not by muscular action. If there were actual suction, such as that by which a mollusk clings to the substrate, circular, or possibly radial, muscles would be required to lift a portion of the tentacle away from the surface to create suction, and in that case release would probably occur as soon as the pull on the tentacle ceased. This is exactly the point which, I think, makes Semon's ('87, p. 283) explanation wrong. He thinks that "die Anheftung geschieht, indem hierdurch beim Nachlassen des Druckes an verschiedenen Stellen zwischen Glaswand und der unebenen elastischen Haut der Tentakel leere Räume oder wenigstens Räume entstehen, in denen das Wasser sich unter geringerem Druck befindet als das umgebende Medium." Since the tentacles remain attached after they cease to be effective in pulling the animal forward, and have to be fairly torn away from their attachment, it seems probable that muscular action plays no part in the adhesion of a tentacle to a surface. This is in accord with the results of anatomical investigations, namely that longitudinal muscles are in all probability the only muscles present in the tentacles (Clark, '07, p. 45). *Synaptulas* will hang so firmly attached to the wall of a dish that they can be shaken and the water quite severely agitated before they will loosen their hold. A *Synaptula* 4 cm. long can crawl up a vertical glass wall carrying in sea-water a piece of iron weighing 30 mgm. in air (ca. 26 mgm. in sea-water) tied to its posterior end.

The peristaltic waves, which assist locomotion through the sea-weed, occur at irregular intervals, having no rhythmic sequence. Each wave starts at the posterior end of the body as a marked constriction some 2 to 3 mm. wide. The constriction passes anteriorly taking about 15 seconds to complete its course in a *Synaptula* 45 mm. long. This rate, about 3 mm. per second, is by no means constant, since in a given specimen waves were found to take 18 seconds at one time and 16.6 at another. Waves appear when a *Synaptula* is moving along a horizontal surface, but not when it is crawling up a vertical wall. They also occur when the animal is defecating. A fairly light mechanical stimulus, such as a touch with a glass rod, causes a local constriction, which often initiates a peristaltic wave if the stimulus is applied to the middle region of the body, but not if applied to the extreme anterior or posterior end. Similarly, local application of various chemicals, such as KCl, NaCl, acetic acid, etc., calls forth constrictions which may initiate a wave if applied to the middle regions of the body. Peristalsis also occurs under the following conditions: when the animals are fed carmine powder; when they are returned to water at 27°C. (the normal) after having been immersed for 3 minutes in sea-water at higher temperatures; when they are placed in calcium-free sea-water.

Since peristalsis appears under such diverse conditions, it is probable that this is a general response given to many kinds of stimuli. At first it was imagined that only excessive stimulation might provoke peristalsis; but this was found not to be the case, since moderate stimuli, either mechanical or chemical, applied to the middle region of the body, nearly always produced this response. It is probably the mechanical stimulation, due to contact, which causes peristalsis when the animal is lying on a horizontal surface or among sea-weeds.

In two instances reversal of direction of the wave was noted. In both cases several peristaltic waves passed completely from the anterior to the posterior end immediately after cutting off the head by a single stroke of the scissors.

When moving through sea-weed the animals often curl the last 5 mm. of the body about a branch and explore their sur-

roundings in all directions. If some twenty or more individuals are placed in a finger-bowl, they at first cling together in a dense wriggling mass. Presently they begin to separate. The tentacles attach to the bottom, then to the sides of the dish, but their posterior ends remain hooked into one another. The tentacles then carry the oral ends up to the top of the water, the posterior ends still holding to one another, until the mass of animals is spread out in a sheet, each individual like a spoke in a wheel. This would seem to indicate a positive reaction to mechanical stimuli at the posterior end of the animal, yet in trials in which this portion was gently touched with a blunt needle, only negative reactions were given.

Although holothurians exhibit the usual radial symmetry common to all echinoderms, they are decidedly more bilateral than other classes of the phylum. The surfaces to which are attached mesenteries that hold the intestine in place, are called dorsal and ventral. The only author who shows that this bilateral symmetry in the apodous holothurians is connected with their behavior is Semon ('87). He found that *Synapta digitata* and *S. hispidia* normally creep with one side, the dorsal, uppermost. In *Synaptula hydriformis*, likewise, there is a tendency to keep one side away from the substrate. This surface is decidedly darker and contains more miliary granules than the opposite one. Clark ('98) found no such difference in specimens of this species which he obtained in Jamaica, but it is certainly very pronounced in those of Bermuda. If a *Synaptula* which is moving horizontally along the bottom of a dish is gently turned over so that the lighter colored side is uppermost, the animal may draw in the tentacles completely, contract the body, roll slightly to the right or left, and then move on again with the dark side up. Usually, however, the animal, when turned over, continues locomotion using the tentacles of the lighter side to pull itself along, rolling over gradually until the dark side again appears uppermost. The average time required for a *Synaptula* to right itself in this way was 45 seconds. In ordinary undisturbed locomotion a few cases were noted where an individual rotated on its long axis, but this was by no means the usual procedure, either when crawling up

the side of a vessel or along the bottom. When a *Synaptula*, in creeping up a vertical wall, reached the surface of the water, it generally began moving horizontally, say to the left, attaching its tentacles more or less in regular sequence, and rolling over, till the lighter side of its body was away from the wall. Then, instead of making a complete revolution and continuing to the left, it would 'roll' back to its starting-point and over to the right, until again the lighter side appeared. It would thus roll back and forth some five or six times before it finally loosened hold and dropped to the bottom. Similarly, while crawling along the bottom of a dish, a *Synaptula* usually rolls back and forth, very seldom moving in a spiral.

Upon dissection this lighter colored side, the one which is kept next the substrate, was found to be that which, for morphological reasons, has been called ventral. Aside from the difference in color and in abundance of miliary granules, differences which are evident in both red and green varieties, there are no external differences between dorsal and ventral surfaces.

The *Holothuria* can be arranged in a series according to increasingly pronounced bilateral symmetry and physiological polarization. One of the lowest members of such a series is *Thyone briareus*, which has tube-feet along all five of its antimeres, and moves with any side forward (Pearse, '08; Mast, '11, p. 211). *Holothuria surinamensis* and *H. rathbuni*, according to Crozier ('14 a, '14 b), are intermediate forms, while *Holothuria captiva* occupies the upper end of the series, since it has tube-feet on the ventral side ('trivium') only and always moves with the oral end forwards (Crozier, '14 b). *Synaptula hydriformis* would rank with *Holothuria captiva*, since, although its bilateral symmetry is not so pronounced, its physiological polarity is most marked.

## 2. *Feeding and digestive movements*

In spite of the fact that this *Synaptula* moves with one particular side next the substrate, its rolling motion brings all the tentacles into play, even those on the dorsal surface. In its natural habitat in sea-weed, all the tentacles are used in locomotion.

tion at the same time, while in a finger-bowl at any given moment the majority of the tentacles wave freely in the water, two or three are attached to the surface, and two or three have been drawn down into the mouth. The animal never remains motionless, but continually moves about, first attaching its tentacles, and then after they are detached, drawing them down into the mouth as if to wipe off particles which might be attached to them. These wiping movements appear to be feeding movements, yet they occur even when no food seems to be present. For this reason Becher ('07) considered them automatic. Quatrefages ('42) found that his *Synapta duvernea* would swallow grains of sand when they were brought to the mouth on the tentacles. Under no circumstances was I able to see particles of any sort transferred from the tentacles to the mouth, though in several experiments carmine grains were caught upon them.

When carmine powder is dropped on the tentacles, they are at once drawn back out of sight into the mouth region, the whole body contracts, and peristaltic waves pass from posterior to anterior. The following observations on one specimen illustrate this behavior.

*July 21. Specimen 4. Collected this morning. Length 4.5 cm.*

- 3.53 p.m. Animal on bottom of dish. Carmine suddenly squirted into mouth.  
Draws in tentacles. Shortens body. Three peristaltic waves.
- 3.55 p.m. Defecates. Three more waves. Climbs to top of dish.
- 3.57 p.m. Drops to bottom. Draws in tentacles. Climbs up.
- 4.00 p.m. Several peristaltic waves.
- 4.01 p.m. Drops to bottom. Two peristaltic waves. Climbs up again.
- 4.05 p.m. Defecates.
- 4.08 p.m. More waves. Drops to bottom.

This is in strong contrast to the ordinary behavior, for a *Synapta* usually remains at the top of the dish for some 10 or 15 minutes without dropping, and no peristaltic waves are to be seen.

If carmine in sea-water is quickly squirted into the mouth while the tentacles are extended, the red grains appear later in the intestine, and finally come out in the castings. In fourteen animals, each about 6 cm. long, carmine appeared in the castings after an interval averaging 20 hours, and varying from 18 hours



in one case to 21 hours in five cases. This means that, unless carmine has an accelerating effect, it takes about 21 hours for the food to pass from the mouth to the anus.

In freshly collected synaptulas one can always see faeces in the intestine at a distance from its anterior end slightly less than half the length of the intestine. The castings are cylindrical masses about 3 mm. long and 0.5 mm. in diameter, and are yellowish white. Often there are two or three attached end to end. Since there are regular rhythmic movements in the whole intestine, there may be some mechanism which determines the form of these castings similar to that of *Stichopus* (Crozier, '16a). Clark ('98, p. 56) states that in specimens from Jamaica "the food consists largely of vegetable matter," diatoms being present in the stomach." Microscopic examination of the contents of the intestine and the faeces of Bermuda specimens failed to show the presence of diatoms, but the empty walls of a small filamentous (red?) alga were abundant. The chromatophores of the alga and all parts inside the cell walls had disappeared in the course of digestion. This difference in food between specimens from Jamaica and from Bermuda is probably due to the greater abundance of diatoms in the West Indies.

The intestine of *Synaptula* shows strongly marked peristaltic waves, which travel from the anterior to the posterior end, a direction opposite to that of the irregularly occurring waves of the body-wall. Since the animal is in constant motion and the integument in most cases is not sufficiently transparent, attempts to time these waves in the normally active animal were not successful, but the average rate of pulsation in the intestine of a *Synaptula* which was held upon a glass slide by weights and whose body-wall was slit so as to disclose the intestine was ten waves in 19 seconds at 27°C. The average rate of pulsation in the intestine after its removal from the body was ten waves in 28 seconds at the same temperature.

The excised intestines assume a constant rate of pulsation within 5 minutes after operation. In two cases, when they were allowed to remain undisturbed, they were found to be pulsating

regularly at the end of 7 hours, though much more feebly than when first dissected out (table 1).

TABLE 1  
*Synaptula intestine in sea-water at 27° C.*

TIME	TIME ELAPSED	RATE: TIME REQUIRED FOR 10 BEATS
10.35 a.m.	Operated	
10.41 a.m.	6 minutes	28 seconds
12.15 p.m.	1 hour 40 minutes	31 seconds
12.45 p.m.	2 hours 10 minutes	30 seconds
3.15 p.m.	4 hours 40 minutes	30 seconds
5.15 p.m.	6 hours 40 minutes	30 seconds

a. *Effect of change of temperature on intestinal pulsation.* The effect of change of temperature on living material has shown than van't Hoff's, or the R. G. T., rule holds good in the organic as in the inorganic world (Pütter, '14). The intestine of *Synaptula* is no exception. The curves in figures 1 and 2, which show the effect of change of temperature on the rate of pulsation, are typical.  $Q_{10}$  is found to have the following values:

$$16^{\circ}\text{--}21^{\circ} = 3.24$$

$$21^{\circ}\text{--}26^{\circ} = 2.56$$

$$26^{\circ}\text{--}36^{\circ} = 1.4$$

These values for  $Q_{10}$  are of the order of magnitude of those for chemical processes, as Crozier ('16 a) found for the rhythmic pulsation of the cloaca of certain holothurians. This series also shows the phenomenon to which Snyder ('11) has called attention, viz.,  $Q_{10}$  is greater for lower than for higher temperatures.

b. *Effect of chemicals on intestinal pulsation.* The effect of chemical agents on rhythmic pulsation has been the subject of considerable investigation (see Crozier, '16 a, for literature). The following experiments on the intestine of *Synaptula* give results which are similar to those obtained by Crozier for *Holothuria*.

The average time for ten beats in artificial sea-water (Mayer, '11, '14), i.e.,  $\frac{5}{8}M$  (100 NaCl + 7.8 MgCl<sub>2</sub> + 3.8 MgSO<sub>4</sub> + 2.2 KCl + 2.5 CaCl<sub>2</sub>) at 27°C. was 29 seconds. This is prac-

tically the same rate as in natural sea-water. Pieces of intestine in the artificial sea-water continued to beat regularly for seven hours, as long as the experiment was continued.

Immersion in  $\frac{5}{8}M$  NaCl immediately caused a severe contraction throughout the entire length of the intestine. However, peristalsis returned in a very few seconds, and was much more pronounced than in normal sea-water. It then gradually be-

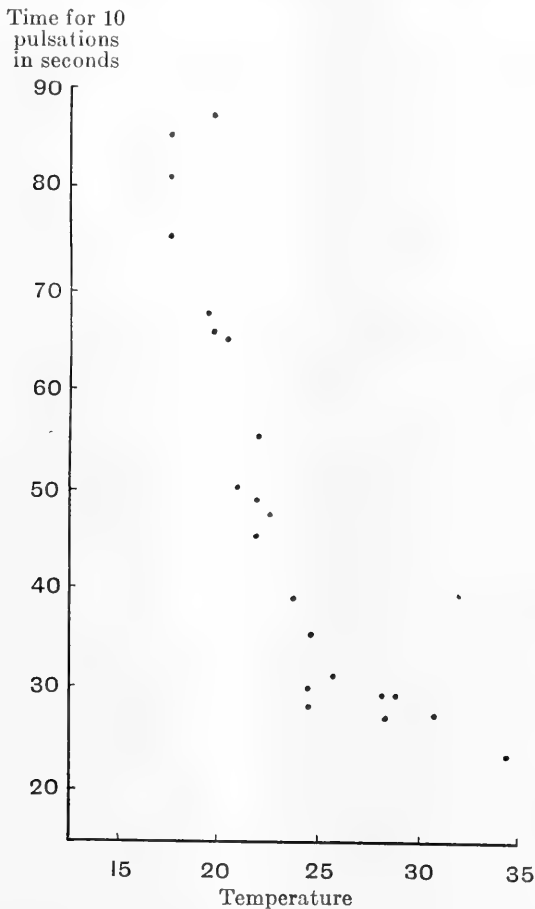


Fig. 1 Ordinates indicate time, in seconds, for 10 pulsations of an excised piece of intestine of *Synaptula*; abscissae indicate temperature, Centigrade. Readings taken on a single specimen.

came feebler and finally (after three minutes) ceased. When the animal was returned to normal sea-water pulsation was not resumed.

$\frac{5}{8}M$  KCl also caused the intestine to contract greatly. No pulsations at all could be detected, nor did it resume pulsation when returned to normal sea-water.

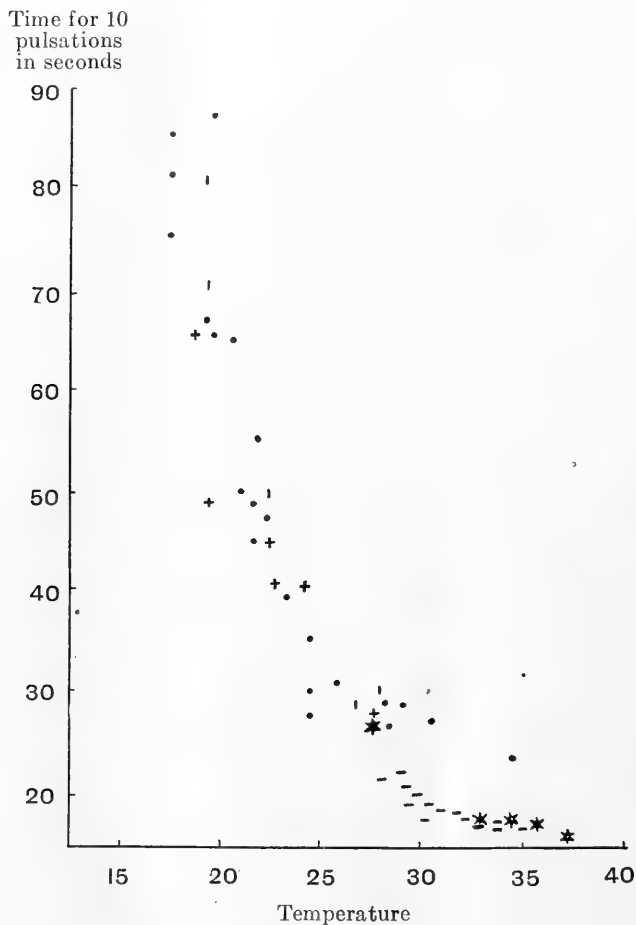


Fig. 2 Ordinates indicate time, in seconds, for 10 pulsations of excised pieces of intestine of *Synaptula*; abscissae indicate temperature, centigrade. Different symbols indicate different individuals from which the pieces of intestine were taken.

The same effect was observed with  $\frac{3}{8}M$   $CaCl_2$ , with  $MgSO_4$  and with  $MgCl_2$ .

In calcium-free sea-water, the intestine writhed and twisted so violently that it was impossible for nearly a minute to count the pulsations. It then became quieter, and also rather opaque, though it beat at its regular rate for at least 45 minutes. After this it twitched irregularly, and finally after about one hour all movement ceased.

The only single component of sea-water, therefore, in which pulsation continues at all is  $NaCl$ , and even here pulsation ceases in a very few minutes after immersion in such a solution.  $NaCl + KCl + CaCl_2$  forms a more 'balanced' solution than  $NaCl + KCl + MgCl_2 + MgSO_4$ , since regular pulsation continues in the former mixture for nearly an hour, but ceases in the latter in some 2 minutes. These results are in perfect accord with those obtained in the study of the cloaca of *Holothuria* by Crozier ('16 a).

There are no histological studies of *Synapta* in which nerves or nerve cells have been shown to be present in the intestine. Its behavior, however, strongly suggests the presence of a nerve net.

### *3. The effects of external stimulation*

*Synaptula*, like the majority of the lower animals, exhibits but few types of reactions. To nearly every external stimulus applied to the body-wall it responds by a local constriction at the point stimulated, or if the stimulus is of sufficient strength, by a shortening of the whole body. The local constrictions are brought about by the contraction of the circular muscles of the regions stimulated and are called forth by such stimuli as touch, light, and chemical agents. If the stimulus is sufficiently vigorous, the constriction may be very deep and initiate a peristaltic wave which moves toward the anterior end of the body. The shortening of the body is, of course, brought about by the contraction of the longitudinal muscles. The antagonistic action of these two sets of muscles and their components in different regions of the body determines, of course temporarily, the shape of the animal. For example, in the contracted state of all

the longitudinal muscles the diameter of the animal may be twice what it is when those muscles are relaxed; the contraction of the longitudinal muscles of one side may produce a curving of the body to that side, either throughout its entire length, or more locally, according to the extent to which the contraction affects the muscles of that side; the contraction of all the circular muscles may produce an extreme elongation of the animal; or, finally, local contraction of circular muscles may produce annular constrictions (a very common condition of all Synaptidae) at irregular intervals.

Stimulation of the very posterior end often causes, in addition to local constriction, a bending away from the source of stimulation, a reaction in which the longitudinal muscles along one side of the body come into play; or the body may be slightly shortened, in which all the longitudinal muscles of the posterior half of the animal are equally contracted. The former reaction is much the more common.

But the most striking reaction which *Synaptula* exhibits is the sudden withdrawal of all the tentacles into the mouth region. This occurs when a moderate stimulus is applied to any of the regions at the anterior end of the animal, e.g., the tentacles, the mouth disc, or the area which extends from the base of the tentacles backward 2 or 3 mm. This sudden turning 'outside in' is the result of very severe contraction of the circular muscles of the head region followed by that of the longitudinal muscles. The contraction of the circular muscles of the head region naturally drives the body fluid backward. When the longitudinal muscles, which are attached to the calcareous ring just below the tentacles, strongly contract they draw the ring, together with the whole head region, back into the body, until the pressure of the body fluid prevents further involution. The posterior ends of animals which have been decapitated present a plump appearance, since the circular muscles for a distance of some 3 mm. back of the cut remain strongly contracted, both closing the wound opening, and forcing the body fluid into the posterior end. A withdrawal of the tentacles is seldom shown unless the anterior end is stimulated.

#### 4. *Other muscular activities*

a. *Blood vessels.* Microscopic examination failed to show any movement in the walls of the dorsal and ventral blood vessels. As Clark ('07, p. 63) states, the blood is moved by the contractions of the muscles in the wall of the alimentary canal. One can see the cells which lie in the lumen of the blood vessel shift back and forth as a peristaltic wave passes down the intestine.

b. *The gonads.* The gonads, however, as Clark ('98, p. 58) also observed, possess the power of independent movement. These movements consist of irregular twitchings, and last for some 30 minutes after removal from the body. Histological study has shown the presence of muscle fibers in these 'genital tubes' (Clark, '07, p. 59).

#### 5. *Bacterial parasites*

Clark ('98, p. 56) found 'no internal parasites' in the synaptulas which he collected at Jamaica. In nearly every one taken at Bermuda there were one to four dark brown or green, almost black, spherical masses, ranging in size from mere specks to 1.5 mm. in diameter. These spheres always lay free in the body-cavity and shifted back and forth with the movements of the animal. When these bodies were crushed and examined under the microscope, they were found to consist of bacteria belonging to the genus *Microcystus*.

Experiments on sensory reaction of this *Synaptula* were conducted on both red and green varieties, but upon comparing records of the animals of the two colors, no difference whatsoever could be found between the behavior.

#### *Summary of Part I*

1. There is no mimicry of its environment by *Synaptula hydriformis*.

2. Attachment of the tentacles is probably by an adhesive secretion alone, not by suction.

3. *Synaptula hydriformis* shows decided physiological polarization, since it always moves with the anterior end foremost, and keeps its dorsal side uppermost.

4. Carmine powder fed to specimens 6 cm. in length appears in the foeces after about 20 hours.

5. The food of Bermuda specimens consists chiefly of a filamentous (red?) alga.

6. The temperature coefficient for the rhythmic pulsation of the intestine obeys the R. G. T. rule.

7. The intestine beats normally in artificial sea-water, and for a much longer time in a balanced solution of Na, K and Ca salts than in any other combination of the components of sea-water. NaCl is the only single component of sea-water in which peristaltic movement of the intestine continues at all.

8. Nearly every Bermuda specimen contains a mass of symbiotic bacteria lying free in the body-cavity.

9. The red and green varieties show no difference in their sensory reactions.

## II. SENSORY PHYSIOLOGY

### 1. *The effect of deforming pressure*

*a. Historical.* The so-called 'touch-papillae' or 'sense-buds' are found in abundance on the tentacles of *Synaptula hydriformis*, but they are scattered over the surface of the body as well. Clark ('07, pp. 47, 48) describes them as "small groups of epithelial cells connected at their inner ends with special ganglia situated at the ends of small nerves, which arise as branches of either the radial or tentacle nerves." "The sensory cells are much more elongated than the ordinary epithelial cells, and the inner end is drawn out into a fiber which connects directly with the small ganglion lying underneath the sense-bud." Retzius ('06), who made a study of the sensory cells of *Synapta Buskii*, finds the same structure and distribution of the sensory cells in this species. Semper ('68, p. 28, p. 153)—not Hamann ('83), as stated by Clark ('98)—seems to have been the first to describe such papillae in Synaptidae, and to suggest that they were



'Tastpapillen.' "Die Lage dieser Papillen macht es wohl ziemlich unmöglich in ihnen etwas anders als Tastorgane zu vermuthen." Semper also tried to show that the calcareous bodies in the dermis of synaptids, especially the anchors, serve as 'touch-organs.' He even compared them to the 'Tasthaare' of the cat. Unfortunately for his idea, he was forced to confess "Bei den Synaptiden . . . ist es mir nie gelungen Nerven bis an die Anker heran verfolgen zu können." Semon ('87) found, as did Quatrefages ('42), that *Synapta inhaerens* could be somewhat roughly rubbed on the skin without eliciting any response from the animal. Since, in addition, chloral hydrate applied to the skin caused a contraction and moving away of the part stimulated, he suggested that the 'touch-papillae' were not entirely composed of touch cells, but contained also gustatory cells. Moreover, since pigment was to be found in these 'touch-papillae,' he thought that some of the cells might serve to receive photic stimuli. Practically all other investigators, however, have been content to ascribe simply a tactile function to these organs.

*b. Observational.* The order of diminishing sensitivity to touch in the different body regions of many of the lower animals is anterior, posterior, middle (Parker, '08; Crozier, '14 a). This order holds for *Synaptula hydriformis*, where the sequence is tentacles, anterior part of the body, posterior part, middle. Table 2 gives a series of reactions obtained by touching the different parts of the body with the blunt end of a needle when the animal was moving back and forth just beneath the surface of the sea-water in a finger-bowl. Similar reactions, though much more variable, were given if the animal was stimulated while moving along the bottom of the dish. In most cases a less vigorous stimulus was required to cause complete withdrawal of the tentacles when the *Synaptula* was at the bottom than when it was near the top of the water.

Of these reactions the only one which can clearly be called positive is the response of the outer surface on the tentacle to a very slight touch. This positive thigmotropism is probably the chief cause of the constant motion of the tentacles. The free tentacles are continually attempting to attach themselves, and

TABLE 2  
*Responses of Synaptula to touch: stimulus applied to various parts of the animal*

STIMULUS	OUTER SURFACE OF SINGLE TENTACLE	INNER SURFACE OF SINGLE TENTACLE	MID-BODY	POSTERIOR END
1. Weak	Adheres to rod	Waves tentacle	None	None
2. Stronger than 1	Tentacle contracts and bends into mouth	Tentacle contracts and bends into mouth	Slight local constriction	Local constriction, bends away
3. Stronger than 2	All tentacles contract, half drawn into mouth	All tentacles contract, half drawn into mouth	Deeper local constriction with wave moving anteriorly	Local constriction and lashing of posterior end
4. Stronger than 3	All tentacles drawn into mouth. Drops to bottom	All tentacles drawn into mouth. Drops to bottom	Very deep constriction. Posterior half of body squirms	Local constriction with shortening of whole body
5. Very strong, (stronger than 4)	Same as for 4	Same as for 4	Drops to bottom with tentacles half drawn in	Drops to bottom with tentacles half drawn in

when once attached they remain so until fairly torn away by the attachment and contraction of other tentacles. The same reaction may also be concerned in food taking. One can readily see the value of this movement should an edible particle come in contact with the tentacle, since further movement toward the object would more surely bring the morsel into contact with the adhesive secretion on the outer surface of the tentacle, whereby a subsequent wiping motion might deliver it to the mouth.

In the reaction to the stimulus applied to the inner surface of the tentacle there is possibly another adaptive movement. The waving of the tentacle after its contact with an edible particle is likely to bring the outer adhesive portion into contact with the object, which might thus be captured and carried to the mouth.

All other reactions are clearly negative, varying in degree of completeness with the strength of the stimulus.

It seems very probable that the touch papillae do have the function of 'tangoreceptors,' but whether they are exclusively such is debatable. Their distribution corresponds with the order of sensitivity, since they are especially abundant on the tentacles and appear to be less numerous on the mid-body than on the posterior end.

Water currents directed on various parts of the body gave results such as those shown in table 3. The method of making the tests was as follows. A pipette bent at right angles was held in a vertical position with its horizontal outlet at a level with the portion of the body to be tested. Water having been drawn up into the pipette to a given height and held there by keeping the finger over the upper opening, the lower end of the pipette was then placed about 2 mm. from the region of the body to be stimulated and the finger suddenly lifted from the pipette. In this way practical uniformity in the strength of the stimuli was secured.

Individuals varied greatly in their responses to water currents. In general, a current from a column 4 cm. high produced no effect on the tentacles or head end. On the mid-body it caused shortening of the body, and on the posterior end shortening of the body with local contractions in addition. A slightly stronger

TABLE 3  
*Effect of water currents on Synaptula*

CURRENT	ON TENTACLES	ON HEAD	ON MID-BODY	ON POSTERIOR END
Weak, column 4 cms.	None	None	Shortens body	Shortens body
Stronger, column 8 cms.	Shortens body	Shortens body	Shortens body	Shortens body
Very strong, water blown through pipette	Partly closes tentacles and drops to bottom	Partly closes tentacles and drops to bottom	Shortens body	Shortens body

current on the tentacles caused shortening of the body only, while a still stronger current caused, in addition to this reaction, a partial withdrawal of the tentacles and consequent dropping to the bottom of the dish. Stronger currents on the mid-body or the posterior end called forth the same reactions as the weaker currents, though more pronounced. When an individual was crawling along the bottom of the dish, no response to any ordinary current was shown, no matter what portion of the body received the stream.

In these experiments with water currents, the tentacles seem to behave contrary to the general rule, since they appear to be less sensitive than the other parts of the body. But this becomes intelligible when one considers that the constant motion of the tentacles must have the same effect as gentle currents impinging on them, and must also cause small currents to continually move over the anterior end of the animal, while the other parts of the body are not subjected to these currents. Once the threshold of stimulation is reached, there is a much more vigorous and complete reaction to a strong current on the tentacles than on other parts, a fact which brings these results into line with the effects of other mechanical agents.

## *2. The effect of gravity and of vibrations*

*a. Historical.* Experiments like those of Kreidl ('93) on *Palaeomon* have shown that those structures which earlier investigators called 'otocysts,' to which we now apply the term 'statocyst,' are, in many of the invertebrates, organs of orientation, since they react to the force of gravity and serve to keep the animal in equilibrium. Reasoning from the well established cases, it is only natural to infer that similar structures, when found in other animals, must serve the same purpose. Such structures have been described in synaptids by Johannes Müller ('50b, p. 226), Baur ('64, p. 46), and others. Hamann ('84, p. 25) considered them larval structures only, and therefore functionless in the adult. They were, however, given the name 'Gehörbläschen,' which implies their relation to the vertebrate organ of hearing.

But the latter organ is concerned with two functions, hearing and maintenance of equilibrium. Quatrefages ('42) could find nothing like audition in *Synapta inhaerens*, and Semon ('87) reported that the synaptids which he examined were 'tone-deaf.' Nevertheless Semon thought it probable that the 'Baur'schen Hörbläschen,' or otocysts, were for the perception of other kinds of vibrations, since the animals were very sensitive to shaking (Erschütterungen). Ludwig und Barthels ('91) agree with Semon that the "Hörbläschen auch an den erwachsenen Thieren als Sinnesorgane funktionieren," though they give no grounds for their belief.

Clark ('98) suggested that in *Synapta vivipara* (*Synaptula hydriformis*) the "otocysts do not function as hearing organs at all, but are of use to indicate the animal's position." He describes these organs as ten in number, "lying external to the radial nerves at the point where they bend backwards over the calcareous ring." They are sacs 60 to 70  $\mu$  in diameter, filled with fluid in which floats or lies a 'cell' which may act as an otolith. In a second paper Clark ('99) records important observations on these organs in *Synapta inhaerens* and *S. roseola*, which he studied in the living condition under the microscope. The structure of the otocyst is the same in these species as in *Synaptula hydriformis*. The otolith changes its position when the position of the otocyst is changed. The makeup of the organ and the behavior of the enclosed otolith are in perfect accord with the requirements of a 'positional organ,' i.e., a surface in intimate connection with nerves on one side, and on the other with a body which changes its relation to that surface whenever the whole structure is moved. But the experiments which Clark performed show merely that his synaptids were affected by change of position. The conclusion that it was the "positional organs" which determined this behavior is an inference only.

Becher ('09) described similar 'Hörbläschen' in *Leptosynaptula bergensis*, and found that they, too, meet the requirements of a 'statocyst.' He also tried tones, and vibrations of lower frequencies, but could observe no response. His statement

“Mit Sicherheit können wir annehmen, dass die Synapten die Stellung ihres Körpers mittels der Statozysten wahrzunehmen instande sind” is, as Buddenbrock ('12) points out, mere speculation, since there is no experimental evidence to prove it. As to Becher's theory of the action of the statocyst, Buddenbrock insists that it is “nicht gut fundiert” and “in die Kategorie der völlig unkontrollierbaren Spekulationen gehört.” From his own experiments Buddenbrock concludes that our present knowledge gives us warrant to assert merely that “bei Synapta die Statozysten lediglich im Dienste einer speciellen Fluchtbewegung stehen, welche die Thiere vom der Oberfläche in die sichere Tiefe führt, und dass ihnen eine sonstige Funktion nicht zukommt.”

*b. Observational.* The following experiments on *Synaptula hydriformis* by no means prove that the statocysts function as positional organs, but they furnish evidence in that direction.

*Synaptula hydriformis*, in contrast to sand-digging forms, is decidedly negatively geotropic. Light is so potent a stimulus that several experiments in which specimens had to go towards the light in order to carry out their usual response to gravity failed to show any results. Experiments conducted in a dark-box open at the top and placed in the center of the room were successful, since in this way the intensity of the light was sufficiently reduced. The animals have no difficulty in creeping up the vertical walls of a glass finger-bowl, therefore a glass plate was used for the trials.

The least angle with the horizon which would induce the *synaptulas* to crawl upwards was  $20^{\circ}$ . At  $18^{\circ}$  they went down or to one side. At  $20^{\circ}$  60 per cent went up, while at  $23^{\circ}$  and above practically 100 per cent went up.

I repeated Clark's ('99) experiment of gently changing the position of the plate on which a *Synaptula* was moving. The plate was held at an angle of  $25^{\circ}$  with the horizon, one edge resting on the bottom of the dish, and when the animal had started well on its way toward the top, the plate was gently turned until an adjacent edge rested on the bottom. This was done so gently that the body of the *Synaptula* was kept in its

former position on the plate, thus avoiding a displacement of the body by gravity which would have influenced its orientation when it fell. In every case the head end turned and bent upwards at right angles to the rest of the body, and then, as its tentacles pulled the *Synaptula* upwards, after orientation had been perfectly accomplished, the body finally fell so that the animal lay in a straight line throughout its entire length.

These results tend to show that the head region is concerned in orientation to gravity. For further proof the 'heads' of specimens were cut off immediately back of the calcareous ring. Both these 'heads' and the decapitated bodies were used in experiments. In every instance the head portions climbed the vertical walls of the vessel, while the posterior portions remained on the bottom in an inert condition. Naturally one can not conclude from such experiments that the posterior ends are unable to orient to gravity, since as a matter of fact they were deprived of their organs of locomotion,—the tentacles,—and therefore might not be able to show any response. To see whether lack of locomotor organs prevented response, I took whole specimens, anaesthetized them in chloretone, and cut off their tentacles. When on the next day they had recovered from anaesthesia, I placed these tentacle-less specimens together with 'headless' ones, anterior ends downward, upon a plate covered with cloth. Although neither the tentacle-less nor the headless animals could climb, they were able to cling to the cloth by means of their anchors. When the plate was inclined at an angle of  $30^\circ$  with the horizon, the extreme anterior ends of the tentacle-less specimens bent around so that their bodies were in the form of a J, while the headless ones remained as they were placed, or at most became slightly bowed. The organs concerned with orientation to gravity therefore can lie only in the anterior end of the animal near the calcareous ring.

To show that this was a true response to gravity, and not to the need of oxygen, I placed several 'heads' in a finger-bowl completely filled with sea-water and inverted in a second dish of sea-water. The rim of the finger-bowl was slightly raised so that the supply of oxygenated water might enter from below.



The 'heads' in every case went straight up the (inside) wall of the bowl directly away from the supply of oxygen.

In contrast to the sand-inhabiting synaptids, like *Synapta inhaerens* (Semon, '87), but in agreement with others, like *Leptosynapta bergensis* (Becher, '07, '09), *Synaptula hydriformis* appears not to respond to vibrations. In individuals placed in a dish resting on an arm of a tuning-fork (run by electricity) whose vibration rate was 256 vibrations per second, no response was observable either when the fork was suddenly started or stopped, or during uninterrupted vibration. Tapping lightly with a metal rod on a dish containing synaptulas failed to call forth a response. Not even when the blow was so severe that there was danger of breaking the dish could any response be detected. Nevertheless a drop of water falling through a distance of 5 cm. on to the surface of water in a finger-bowl containing synaptulas was sufficient, in most cases, to cause partial closing of the tentacles and a dropping of the specimens to the bottom of the dish. A fall of a single drop of water through less than 5 cm. produced, in general, no effect. One individual did not respond until the drop had fallen through 10 cm.

These experiments show (1) that *Synaptula hydriformis* does not respond to vibrations of relatively high frequency, (2) that it is distinctly negatively geotropic, and (3) that the organs which respond to the force of gravity are located at the anterior end of the animal very near the calcareous ring. Now, this is exactly the position where the statocysts are to be found. These organs are so small and in such a location that the difficulties of removing them for experimental purposes are great. Indirect evidence of the kind brought out in the above experiments must therefore decide the question as to whether the statocysts are truly organs of orientation.

Since Clark ('98) has shown that the structure of these statocysts meets the requirements for 'positional organs,' and since my experiments show that the animal does orient to gravity, and that the organs concerned with this response can lie only in the region where statocysts are to be found, I think it safe to

conclude that the statocysts are the organs concerned in the orientation of *Synaptula hydriformis* to gravity. My experiments show further, that *Synaptula hydriformis* does not respond to such vibrations as are said to produce a tone when they reach the ear of man.

### 3. *The effect of light*

*a. Historical.* One of the earliest observers, and probably the first to record physiological experiments on synaptids, was Quatrefages ('42). He tried the effect of light, [sounds] vibrations, mechanical and other stimuli upon his *Synapta duvernaea* (*S. inhaerens*) and states (p. 29), "Je n'ai pu reconnaître chez elles la moindre trace de vision, proprement dite, non plus d'audition ou d'odorat." Nevertheless he thought the activity of these synaptas was reduced during the daytime. When he exposed them to the rays of his lamp concentrated by means of a lens, "elles en étaient évidemment incommodé esquelle que fût la partie du corps placée au foyer, mais surtout lorsqu'un dirigeait cette lumière sur les tentacles; on les voyait alors se détourner et quelquesfois se contracter et revenir en partie sur elles meme" (p. 28). His physiological observations were evidently forgotten for some time, for the discussion of sense organs in synaptids became one of merely anatomical relations.

The first description of the 'eye-spots' of synaptids was given by Müller ('50 b, p. 226; '52, p. 16). Semper ('68, p. 152) concluded that the sense organs which Müller described, the 'Augenflecken' and 'Gehörblasen,' were both somewhat problematical. "Ob die von Müller entdeckten Augenflecke der Synapten wirklich als Sinnesorgane, analog den mit dem Nervensystem in Verbindung stehenden Pigmentflecken verschiedener Thiere, aufzufassen sind, ist noch nicht zu entscheiden." Baur ('64) found that in *Synapta digitata* the pigment masses which lie at the bases of the tentacles are not different from those scattered over the surface of the body, and Hamann ('84, p. 26) agreed with Baur that they should not be considered as 'Augenflecke,' but simply as 'Plasmawanderzellen.' Semon

('87) seems to have been impressed by Quatrefages' observations and tried several experiments on *Synapta inhaerens* and *S. digitata*. He was, however, unable to discover any reaction to photic stimuli. Sudden strong increase of light or sudden shading gave no results. He was therefore induced to agree with Baur and Hamann that these pigment spots were not true eye-spots.

Ludwig und Barthels ('91), on the contrary, are emphatic in their statement that the so-called eyes in *Synapta vitatta* and *S. vivipara* (*Synaptula hydriformis*) are undoubtedly sense organs. Probably their assertion is based on morphological grounds, though they do not so state. Clark ('98), too, says that "there can be little doubt that in *Synaptula vivipara* these eyes are actually of service as light-detecting organs," though he reports no experiments to prove his position, merely remarking: "That this covering (the mesodermal layer surrounding the nerves to the eye-spots) may be affected by light is probable, for its color is due to the pigment it contains." Yet he also states, "There is no reason to assume that the pigment in other parts of the body is any different from that around the eyes."

*b. Observational.* It is a fact that in several of the lower animals, eye-spots, so-called from morphological considerations, have been clearly shown not to function as eyes at all (Nagel, '96, p. 34). In other cases, although eyes are present as true photoreceptors, other parts of the covering of the body are also sensitive to light (Parker, '03). Whether the eye-spots in *Synaptula hydriformis* are truly special photoreceptors, I have as yet been unable to ascertain. One thing, however, is certain, the whole surface of the body is sensitive to light.

This *Synaptula* is decidedly negatively phototropic. When placed facing a window in the daytime or before an electric lamp at night, it at once turns from the light and moves to the opposite side of the dish in a fairly straight line. The changes of body position in this orientation are exactly those figured by Crozier ('14 b, p. 12, fig. 1) for *Holothuria captiva*. Consequently Mast's ('11, p. 211) sweeping statements—"The Echinoderms are peculiar in that they can move with any side

ahead," "The lack of orientation in moving from a source of light is much more striking in the Holothurians," and "there is no orientation in these animals"—hold for species like *Thyone briareus* (Peare, '08), but not for *Holothuria surinamensis* (Crozier, 14 a), *Holothuria captiva* (Crozier, '14 b), or *Synaptula hydriformis*.

If the heads of synaptulas are cut off just back of the tentacles, they, too, will move to the side of the dish away from the source of light, whether it be the sun (direct or indirect light), or an electric (32 candle power) lamp. When the posterior portions of these headless animals are placed with their anterior ends in the sunlight, they also show response to the light by bending their cut ends away from the light, so that the body becomes bent into a U. If, however, these posterior portions are placed some distance (five feet) from a window on the side of the room opposite that where the sun is shining in, or near an electric lamp, they do not orient to the light, but remain practically as they were placed. If the tentacles only are cut off (the eye-spots being left intact), the animals bend their anterior ends away from the light, and gradually bring their posterior ends around until the animal lies in a straight line headed away from the source of light. In this respect they differ from the headless synaptulas, since the latter remain bent. Posterior headless pieces placed in the dark with their cut ends arranged in a line, were found after 30 minutes to be in practically the same position as that in which they had been placed at the beginning of the experiment; therefore the bending away from the light of the headless portions is a true photic response, and not the result of injury.

Spots of light thrown on different parts of the body of *Synaptula* showed that all parts were sensitive to light. Experiments were first tried in which the synaptulas were placed on the bottom of a dish containing 20 cm. of water. A spot of sunlight, concentrated by means of a lens of 25 cm. focal length, was thrown on the various parts of their bodies. The response, no matter what portion of the body was illuminated, was so violent, that it seemed almost impossible that it

could result from photic stimuli alone. It was found that if a similar spot of light was thrown on a thermometer placed in the same situation, there was an immediate rise from 27° to 34°C. A column of sea-water 20 cm. high was therefore not an efficient heat screen. Nevertheless, other experiments had shown that synaptulas did not respond to a sudden change of temperature from 27° to 34°C. It was thought best, however, to eliminate any possible stimulation from heat rays. Accordingly the animals were placed in a small dish within a box whose sides were painted black. Over a hole in the top of the box was placed a glass tank containing sea-water to the depth of 27 cm. The sunlight came through the water, passed down into the box, and was concentrated on the synaptulas by means of small hand lenses of 2 and 2.5 cm. focal length. When this very small spot of light was allowed to fall on a person's finger, he was unable to perceive any rise in temperature. If the light was thrown on a thermometer, no change could be noted. The results obtained under these conditions are given in table 4.

TABLE 4  
*The effect of local stimulation by light*

REGION STIMULATED	RESPONSE
Base of tentacles	Draws tentacles in. Shortens whole body. Local constriction
Body anterior to middle	Local constriction only
Body posterior to middle	Local constriction only
Posterior end	Local constriction. Shortens whole body. Bends posterior end away

All parts of the body of *Synaptula* respond to local stimulation by light of the intensity used in these experiments. The middle portion of the body gives less vigorous response than either the head or the posterior end. The order of sensitivity of parts is, therefore, the same as that for other holothurians (Crozier, '14 a, 14 b; cf. also Parker, '08).

Headless animals were tested in the same way and were found to give reactions similar to those of entire animals. The

severed heads, however, behaved somewhat differently. When the spot of light from above was thrown on them, they at once turned and moved off at approximately a right angle to the direction in which they were first moving. Only once did a head attempt to draw in its tentacles; that was after the spot of light was kept focussed on it as it moved about. At least 10 seconds of constant stimulation was necessary to produce this reaction, whereas in entire animals this response was practically instantaneous.

The results of these experiments make it seem doubtful if the 'eye-spots' are 'light-detecting' organs. For, first, the whole body is sensitive to light, and, secondly, parts of the body without eyes are able to orient away from a source of light. Nevertheless, one must take into account the fact that in headless, and therefore eyeless, specimens orientation did not occur in light of less intensity than direct sunlight, while the heads of the same animals, which contained the eyes, did orient under the same conditions. This is simply further indication that the head end is more sensitive than other parts of the body, and in no way does it prove that the presence of the eye-spots is responsible for the greater sensitivity. Parker ('08) found that the anterior end of *Amphioxus*, which contains a so-called eye-spot, was more sensitive to light than either the middle or posterior part of the body. Yet he showed that this eye-spot was not a photoreceptor, because light focussed upon it elicited no response from the animal. The true photoreceptors in *Amphioxus* were certain organs distributed along the nerve cord, more of them being found at the anterior end, the region of greater sensitivity. This has been confirmed and further demonstrated by Crozier ('17). Similarly in *Synaptula*, the whole surface of the body is sensitive to light, the anterior end being more so than the other parts, perhaps in spite of, rather than because of, the eye-spots.

#### 4. *The effect of heat*

*a. Historical.* Few investigations have been made upon the reaction of holothurians to heat. Crozier ('14 a) found that

none of the several holothurians which he tested seemed to be "equipped with anything which might properly be called a temperature sense." Kafka ('14, p. 214) dismisses the subject with little more than the statement, "Die Holothurien scheinen für Temperaturänderungen ziemlich unempfindlich zu sein."

*b. Observational.* *Synaptula hydriformis* evidently possesses only a feeble temperature sense. If a half cubic centimeter of water at any temperature between 14° and 42° C. is allowed to flow gently from a pipette upon the tentacles of a *Synaptula* immersed in sea-water of a temperature of about 27°C., there is no response. But water at 10° or less, and at 46° or more thus applied, invariably causes the animal to respond. Table 5 gives the percentages of responses for these and other temperatures.

TABLE 5

*The effect of 0.5 cc. sea-water at different temperatures flowing on tentacles*

TEMPERATURE	PER CENT OF RESPONSES
8°	100
10°	100
12°	77
13°	65
14-42°	0
43°	30
45°	88
46°	100

A second method used in testing the temperature sense was that of carefully lifting a *Synaptula* out of water at 27°C. (the normal) and suddenly immersing it in water at the desired temperature. The animal was allowed to remain 3 minutes at the given temperature and then carefully returned to water at 27°. Table 6 gives the results of such trials.

Here the limits between which there were no responses were 20° and 40°. Beyond these limits one or more vigorous contractions of the whole body were given, followed by relaxation at the higher, and partial contraction at the lower temperatures. To produce death in 3 minutes a temperature of 46° was necessary.

TABLE 6

*The effect of suddenly transferring Synaptulae from sea-water of 27°C. to that of different temperatures, leaving them 3 minutes, and then returning them to that of 27°*

TEMPERATURE	EFFECT OF CHANGE	EFFECT OF RETURN
14°	Vigorous general contraction of whole body. Constriction back of tentacles. Keeps moving	Behaves normally after lengthening of body
15°-18°	Same as above	Same as above
19°	Less vigorous contraction. No constriction	Same as above
20°-39°	No response	No response
40°	Three contractions of whole body. Posterior remains contracted. Tentacles still move	2-3 peristaltic waves. Behaves normally
43°	Same as at 40°, but entire relaxation after 2 minutes	Same as from 40°
44°	More vigorous contraction. Anterior end remains contracted. Posterior relaxes	Same as from 40°
45°	Vigorous contraction. Followed by complete relaxation	Vigorous contraction. Peristaltic movements in posterior end only. Anterior end killed
46°	Feeble contraction. Died at once	No recovery

Local application of heat was tried with negative results. A glass tube of small bore was bent into a narrow U, the ends of which passed through a cork for convenience in holding. By means of a siphon and rubber tubing, hot water was continually passed through the U-tube. The mean between the temperature at which the water entered and that at which it left the tube was taken as the temperature at the middle region of the system where the U-tube was situated.



Water at 27° was first run through. It was found that there was no response when the tube was held near a *Synaptula*, or even when it touched the animal, if this was done with sufficient care, except that when in contact with the tentacles they would attach themselves to the tube and the animal would attempt to climb along the tube. Water at 47° and even at 50° failed to show any effect, although both temperatures are above the killing point, and the water surrounding the U-tube must have been raised considerably above 27°. Indeed, the action of the tentacles was the same as when the water at 27° was running through it, since they attached to the tube in the same manner.

From these results one must conclude that the temperature sense of *Synaptula hydriformis*, while not entirely absent, is very poorly developed and probably not located in any particular regions. Since in its natural habitat the animal is not subjected to sudden or great changes of temperature, it is not surprising to find this sense practically lacking.

#### 5. *The effect of chemical agents*

*a. Historical.* Chemical agents may produce in vertebrates the sensation of smell or taste according as they stimulate the olfactory or gustatory organs, or they may affect the free nerve endings of the epidermis, the receptors for the common chemical sense (Parker, '12; Crozier, '16 a). These three kinds of receptors are found in aquatic as well as land vertebrates (Sheldon '09; Parker, '12). Among aquatic invertebrates some mollusks have been shown to have separate senses of smell and taste (Kafka, '14, p. 269), as proven by topographical experiments and by the difference in histological makeup of the organs. Copeland's ('17) recent experiments on snails give further proof of the separateness of these senses in mollusks.

Among the echinoderms the starfish was thought by Romanes ('85) to have a sense of smell located in the ventral surface of the rays. More careful experiments by Jennings ('07) on the starfish *Asterias forreri* showed that the pedicellariae on the dorsal surface responded to the juice of crab-meat held a little above the surface of the animal. It is therefore probable that

there is present only a general chemical sense in the starfish and that this is distributed over the whole surface of the body.

The reactions of holothurians to chemical agents have been studied intensively in only one species, *Holothuria surinamensis* (Crozier, '14 a), while only scattered observations on the chemical sense of synaptids are to be found in the literature. Quatrefages ('42) placed specimens of *Synapta inhaerens* in solutions of opium to quiet them, but was unsuccessful in attaining this end. He also found that fresh water had practically no effect on this species, and that pieces of the animal would remain alive in it for some eight days. Synaptids were thought by Semon ('87) to have a very keen sense of smell and taste, which he located in the cup-shaped outgrowths on the inner face of the tentacles. These organs had been noted by Quatrefages ('42, Planche 4, fig. 1 and Planche 5, fig. 3), who called them 'ventouses.' These are the same structures as Müller's ('50) and Baur's ('64) 'Saugnäpfe' and Hamann's ('84) 'Sinnesknospen.' Semon's reason for locating these special senses in these organs are as follows: (1) pieces of food attached to the ends of the tentacles as they are bent down to the mouth must pass by these cups; (2) histological study of the structure of these organs shows—by far the more weighty evidence in his opinion—that they are lined with cilia and that nerves pass from them to the tentacle nerves. Such 'proof' is far from conclusive. Moreover, when Semon makes the statement that bringing a strong-tasting substance, such as chloral hydrate, into the neighborhood of the tentacles causes a most vigorous reaction, one realizes that he is speaking of what we now call a common chemical sense, and nothing so definite as smell and taste. To account for the fact that this 'strong-tasting' substance, chloral hydrate, also stimulated any region of the body to which it was applied, he had to suppose that the 'touch-papillae' contained taste cells, or that such cells were scattered over the surface of the body "to inform the animal as to the good or bad quality of the sand." Clark ('99) found that *Synapta rosea* and *S. inhaerens* moved away from rank smelling substances placed in the water near them, even though these substances were not touching the

animals. Clark ('98, '07) has followed Semon's example in calling the cups on the tentacles gustatory organs. He remarks, "There seems to be little doubt that these cups serve as organs of either taste or smell, although the evidence is not conclusive."

There are, however, synaptids which lack these cups, among them is *Synaptula hydriformis*, and yet this species is very sensitive to chemical agents. The function of the 'gustatory cups' therefore seems to be still unsettled. Clark ('07) states that synaptids which possess eye-spots never have gustatory cups, and vice versa. Upon the ground that the functions of these two organs are those suggested by their names, the relation of the presence of one of these organs to the absence of the other is hard to explain.

*b. Observational.* *Synaptula hydriformis*, like the majority of aquatic animals, is sensitive to chemical agents over the entire body. The most vigorous reactions are given when the tentacles or the head end are stimulated. For this reason experiments to determine the limits of sensitivity were performed by allowing 0.5 cm. of a solution of a given reagent to flow down the inside of the vessel for 2 to 3 mm. till it reached the surface of the water directly above the tentacles of an animal moving about just under the surface. More constant reactions were obtained when the animal was tested in this position than when it was crawling on the bottom of the dish. Trials with ordinary sea-water were made before each experiment, and often between successive exposures to the chemical solution. With rare exceptions there was no response to sea-water. Ten minutes was allowed between successive trials, so that fatigue of the sense organs might not occur, and so that the animal might resume its position at the top of the dish if it had dropped to the bottom. In general the same chemical agent was not used in two consecutive trials in order to avoid a possible accumulative effect. The following record is typical, x denoting no response.

*July 25. Specimens collected this morning*

	SPECIMEN				
	No. 1 (Green)	No. 2 (Green)	No. 3 (Red)	No. 4 (Red)	No. 5 (Green)
<i>M</i> /100 NaCl....	x	x	x	x	x
<i>M</i> /100 KCl.....	x	x	x	x	x
<i>M</i> /100 Na acetate.....	x	x	x	x	x
<i>M</i> /10 KCl.....	Draws in tentacles, drops to bottom of dish	Draws in tentacles, drops to bottom of dish	Animal drops to bottom of dish	Animal drops to bottom of dish	Draws in tentacles, drops to bottom of dish
<i>M</i> /10 Na <sub>2</sub> SO <sub>4</sub> ..	x	x	Drops	x	x
<i>M</i> /10 Na acetate.....	x	x	x	x	x
<i>M</i> /20 KCl.....	Drops	Drops	Drops	Drops	Drops
<i>M</i> /10 MgCl <sub>2</sub> ....	x	x	x	x	x
<i>M</i> /10 cane sugar.....	x	x	x	x	x
<i>M</i> /10 MgCl <sub>2</sub> ....	Draws in tentacles, drops	Same as no. 1	Same as no. 1	Same as no. 1	Same as no. 1

In table 7 are given the limiting concentrations of the various classes of chemical agents. These were determined by making at least two trials on each of five or more different individuals. If, with a certain concentration, definite responses were obtained in half the number of trials, this concentration was considered to be the limit. Upon trying double this concentration it was invariably found that a decided response to every trial was given by every individual, and that to half this concentration no reactions at all were shown. Table 8 has been compiled for purposes of comparison.

*Synaptula hydriformis*, in contrast to *Synapta inhaerens* (Quatrefages, '42), is very sensitive to fresh water. Indeed fairly slight changes of osmotic pressure call forth a very decided reaction. 8 cc. ordinary sea-water + 2 cc. rain water causes a vigorous reaction, while 9 cc. sea-water + 1 cc. rain

gives none. If the concentration of the salts in sea-water is taken as  $5/8 M$ , (Mayer, '11, '14), then the former dilution corresponds to  $1/2 M$ , i.e., a change of  $1/8 M$  is sufficient to cause a reaction. On the other hand, 6 cc. sea-water + 4 cc. sea-water

TABLE 7  
*Limiting concentrations of chemical agents*

	REAGENT	RESPONSE TO	NO RE- SPONSE TO
Sweet.....	{ Glycerine	$1/2 M$	$1/4 M$
	{ Sugar (cane)	$1/2 M$	$1/4 M$
	{ Saccharine	$1/200 M$	$1/400 M$
Sour.....	{ Acetic acid	$1/400 M$	$1/800 M$
	{ Hydrochloric acid.	$1/600 M$	$1/900 M$
	{ Oxalic acid	$1/200 M$	$1/400 M$
Anaesthetics.....	{ Alcohol	$1/5 M$	$1/10 M$
	{ Ether	$1/100 M$	$1/200 M$
Alkali.....	Ammonium hydrate	$1/200 M$	$1/400 M$
Salts.....	{ NaCl	$1/4 M$	$1/8 M$
	{ KCl	$1/40 M$	$1/50 M$
	{ KBr	$1/40 M$	$1/80 M$
	{ $CaCl_2$	$1/20 M$	$1/40 M$
	{ $MgCl_2$	$2/5 M$	$1/5 M$
	{ $NaSO_4$	$1/8 M$	$1/10 M$
	{ KI	$1/80 M$	$1/160 M$
	{ $NH_4Cl$	$1/15 M$	$1/20 M$
	{ $NaC_2H_3O_2$	$1/10 M$	$1/20 M$

TABLE 8

*Minimum concentrations of chemical agents which will call forth responses when applied to the mouth of certain vertebrates or the tentacles or mouth region of certain invertebrates*

	HCl	NaOH <sup>1</sup>	NaCl	QUININE	SUGAR
Amphioxus (Parker, '12)....	$1/500 M$				N.R. <sup>1</sup>
Ammocoetes, (Parker, '12).	$1/1280 M$	$1/1000 M$	$1/40 M$	$1/640 M$	N.R.
Mustelus, (Parker, '12)....	$1/75 M$	$1/10 M$			N.R.
Amiurus (Parker, '12).....	$1/20 M$	$1/100 M$	$1/50 M$	$1/150 M$	N.R.
Man (Parker, '12).....	$1/1000 M$	$1/400 M$	$1/50 M$	$1/25,000 M$	$1/50 M$
		(KOH)			
Holothuria (Crozier, '14 a).	$1/500 M$	$1/500 M$			
		( $NH_4OH$ )			
Synaptula hydriformis.....	$1/600 M$	$1/200 M$	$1/4 M$	$1/10,000 M$	$1/2 M$

<sup>1</sup> N.R. signifies that there was no reaction.

which has been evaporated to one-half its normal volume gives a reaction, while 7 cc. ordinary sea-water + 3 cc. of the concentrated sea-water fails to call forth a reaction. The former concentration corresponds to  $7/8 M$ . The range of concentrations between which osmotic pressure does not serve as a stimulus is therefore  $1/2 M$  to  $7/8 M$ . The significance of these limits will appear in the discussion of the effect of certain agents such as sugar and glycerine.

Since changes in osmotic pressure caused such marked reactions, it was impossible to make up solutions in pure water.  $5/8 M$  NaCl or KCl in pure water produced violent reactions, from which it required several minutes for the individual to recover. Solutions were therefore made up in sea-water. Solid agents were dissolved directly in sea-water, and the normality of the solution was reckoned as if pure water had been used. Liquids like HCl were added in double the concentration desired to sea-water evaporated to one-half its normal volume. It is by no means claimed that these limits are as accurate as the table suggests. The first drops of the solution must necessarily be much diluted by having to pass through several millimeters of sea-water before reaching the Synaptula. The last part of the discharge must, however, flow against the animal in approximately its original concentration. If these limiting values err, they do so in being too high; still they are significant for comparing the effects of different agents on this one species, even if they may be less so for comparing the degree of sensitivity of Synaptula with that of other animals.

Parker ('12, p. 228) considers that aquatic vertebrates will not respond to that class of chemical substances which in man produces the sensation of sweetness. Crozier ('14 a, p. 289) believes that some sugars may prove stimulating, since *Holothuria surinamensis* responds to maltose and glycerine. The  $1/2 M$  cane sugar and glycerine solutions to which Synaptula responds (table 7) have a total concentration (salts in sea-water plus sugar or glycerine) of  $9/8 M$ , while the  $1/4 M$  solution, to which it does not respond, has a total concentration of  $7/8 M$ . It is evident therefore that we are dealing in the case of the

reactions of *Synaptula* to sugar and glycerine simply with an osmotic pressure phenomenon, and not with a response comparable to our gustatory sensation of sweetness. The close correspondence of the limiting concentrations for stimulation by increased osmotic pressure (1) when the sea-water is concentrated by driving off water and (2) when sugar or glycerine are added, shows that the method is at least consistent with itself.

Saccharine, however, proves to be very stimulating. Whether *Synaptula* actually experiences a sweet sensation from a solution of saccharine, it is impossible to state. In man saccharine is sweet to the tip of the tongue, but rather bitter to the back of the tongue. This difference is more pronounced in parabrombenzoic sulphinid, a bromine substitution product of saccharine (Howell, '16, p. 298). It may be possible that in *Synaptula* only a bitter (alkaloid) effect is perceived when the animal is stimulated by a solution of saccharine.

Of the three acids tried, HCl caused a response at the lowest, acetic at a slightly greater, and oxalic at a still greater concentration. Neglecting the suppression of ionization of HCl due to the presence of Cl ions already in the sea-water,—the effect of which is very slight (Chick, '13, p. 333),—there would be in 1/600 *M* HCl a hydrogen ion concentration of about 0.0016 *N*, since HCl at that dilution is practically wholly dissociated. But acetic acid at 1/400 *M* is slightly less than 8 per cent dissociated, therefore the concentration of the hydrogen ion in the limiting concentration of HCl for stimulation is some 8 times that in the limiting concentration of acetic acid. This is exactly the phenomenon to which Crozier ('16 a) refers as occurring in human taste. He suggests, as an explanation of this anomaly, that "potentially ionizable hydrogen" is present "within the undissociated acid molecules, though secondary ionization may also play a part."

Two alkaloids only were tried, strychnine and quinine. The former is the more stimulating (cf. Crozier, '14 a). The sensitivity of *Synaptula* to these reagents is remarkable. It responds to a solution of 1/10,000 *M* quinine; but even in man the limit is only 1/26,000 *M* (Parker, '12). After stimulation

with strychnine and especially with quinine, *Synaptula* responds vigorously to very weak stimuli of any sort. An application of 0.5 cc. of 1/10,000 *M* quinine will for hours afterwards render useless all further experimentation on an individual, and after one or two drops of 1/1000 *M*, the slightest touch on the tentacles is sufficient to cause a sudden and violent withdrawal of all the tentacles into the mouth region, even twenty-four hours after the application of the quinine. Henri ('03) found that after an application of a weak solution of strychnine sulphate to the holothurian *Stichopus regalis* there was a very marked augmentation of sensitivity. Jennings ('07, p. 69) found that the starfish *Asterias forreri*, after stimulation by certain chemical substances, gave a much readier response to mechanical stimulation. Crozier ('14 a, p. 285) obtained similar results on *Holothuria*. Even in man this is true, since in cases of strychnine poisoning "the slightest stimulus, such as a faint noise, a draught of air, etc., is sufficient to throw the patient into general convulsions" (Lickley, '12, p. 17; cf. Herrick, '15, p. 65).

Not only does this marked increase in the sensitivity of *Synaptula* to weak stimuli follow after application of alkaloids, but also after excessive or severe stimulation, such as keeping them in water at 42°C. for several minutes.

If the kations of the chlorides used in the experiments on stimulation with salts are arranged in a progressive series beginning with Mg, which is the least stimulating, and therefore has the greatest limiting concentration, we find the order, Mg, Na,  $\text{NH}_4$ , Ca, K, this being the usual so-called liotropic series (Höber, '11, p. 497; Crozier, '14, p. 289, '16 a, p. 345). But since 1/2.5 *M*  $\text{MgCl}_2$  in sea-water has a total concentration of 1 *M*, the response to this salt is probably one to increased osmotic pressure only.

An anion series where Na was the kation of each salt runs as follows: chloride, sulphate, acetate (Höber, '11, p. 487). Although the limiting concentrations of KCl and KBr appear the same in the table, KBr is slightly the more stimulating of the two, since the number of reactions to 1/40 *M* KBr was at least 50 per cent greater than to 1/40 *M* KCl. This is also



indicated by the fact that the concentration of KCl which just failed to stimulate *Synaptula* was  $1/50\ M$ , while for KBr it was  $1/80\ M$ . Therefore we find this series to be: chloride, bromide, iodide (Höber, '11, p. 487).

No attempt was made to carry out experiments on 'balanced' solutions, since calcium-free sea-water proved stimulating. In 60 trials with calcium-free sea-water, conducted at different times during the summer on many different specimens of *Synaptula*, 31 resulted in vigorous responses, 11 in slight responses, and 18 in no response. There was no reaction to magnesium-free sea-water. The effect of calcium-free sea water is plainly shown in the following record. The individuals were gently transferred from ordinary sea-water and totally immersed in the calcium-free sea-water. Similar transference from normal sea-water to normal sea-water, carried out as a check, caused no reaction.

*July 26*

- 4.15 p.m. Five individuals transferred to calcium-free sea-water. All five at once contract body, then draw in tentacles violently, then straighten out.
- 4.16 p.m. Violent peristaltic waves of body wall in quick succession. No attempts to climb the sides of the dish. Squirm about on bottom. Three individuals defecate.
- 4.20 p.m. All much contracted, lying motionless on bottom.
- 5.10 p.m. Do not respond to slight touch, or even vigorous poking, or to solution of  $1/400\ M\ HCl$ .
- 5.15 p.m. Restored to normal sea-water.
- 5.20 p.m. Four perfectly recovered. Climb walls. Respond to touch, etc. Others still motionless.

*July 27*

- 9.30 a.m. All five animals in perfect condition. Respond to touch, etc. Calcium-free sea-water therefore acts as an anaesthetic.

Local application of solutions of chemical agents to different parts of the body gave characteristic results. Fifteen trials where 0.5 cc.  $1/10\ M\ KCl$  was allowed to flow very gently from a capillary pipette ending about 2 mm. from the regions to be stimulated gave the following:

1. On tentacles. Tentacles close and animal drops.
2. On mid-body. Local contraction, which initiates a peristaltic wave.
3. On posterior end. Local contraction. Posterior end lashes about from side to side. In one case the animal contracted its tentacles and dropped.

The same results as for KCl was produced by  $1/10$  M  $\text{CaCl}_2$ , Na acetate, or Na citrate.

$1/10$  M NaCl,  $\text{MgCl}_2$ , or  $\text{Na}_2\text{SO}_4$  gave the same results as a current of ordinary sea-water, i.e., were not stimulating. To be more certain that the effects of  $1/10$  M KCl,  $\text{CaCl}_2$ , Na acetate, and Na citrate were not due to currents, a crystal of oxalic acid was held in forceps just over a Synaptula. When the crystal was above the tentacles they were at once drawn in violently; if over any other portion of the body, there occurred deep local constriction, and the whole body posterior to the stimulated region became greatly contracted; if over the extreme posterior end, local contraction took place, a shortening of the whole body, and a lashing about of the posterior end.

Synaptula hydriformis is therefore very sensitive over its entire body to changes in osmotic pressure and to chemical agents, more so at the anterior end, less at the posterior, and still less in the mid-body regions. It responds to the same categories of chemical agents as do the vertebrates (Crozier, '14 a), i.e., acids, salts, sugars(?), alkaloids, alkalis, and anaesthetics.

#### 6. General discussion

It cannot, as yet, be stated whether there have been developed in this holothurian separate sense organs for the different classes of chemical stimuli, or indeed for chemical stimuli in general as distinct from tactile stimuli, etc. The former seems highly improbable, and the latter perhaps doubtful. Aside from the eye-spots and the otocysts, only one type of sensory cell has been described in synaptids (Clark, '98, '07; Retzius, '06), namely the usual invertebrate bristle cell terminating at its deep end with a fiber which connects with a nerve net. The presence of such cells in the vertebrate olfactory epithelium has led Parker ('12) to consider this as the primitive type of sense organ carried over from the invertebrates to the vertebrates. Others (Herrick, '08; Sheldon, '09) have considered the free nerve terminations, which serve as the receptors for the common chemical sense in the vertebrates, to be the primi-

tive type. In support of the former view, it may be urged that not only is the histological structure of the vertebrate olfactory sense cell the same as that of the invertebrate bristle cell, but the physiological characters of the two kinds of cells are similar. (1) The nature of the stimulus is the same, viz., chemical substances, (2) they are 'distance receptors,'<sup>1</sup> and (3) a very minute amount of substance serves as a stimulus.

Nagel ('94) has advanced the idea of the existence of 'universal sense organs' in the invertebrates, i.e., organs which, instead of being capable of receiving stimuli of only a limited range, can receive stimuli of all sorts. In the course of evolution from the lower to the higher forms, certain of these 'universal sense organs,' according to this view, became more restricted in their sensitivity and responded to fewer and fewer classes of stimuli, until finally such specialization occurred as is shown in the present condition of man, where there are no recognizably 'universal sense organs' but some twenty (Herrick, '15, p. 74) special senses.

It may be that *Synaptula* affords an example of an animal in which these universal sense organs exist. The methods of determining whether or not sense organs are specialized to receive one class of stimuli only are (1) to apply these stimuli on separate portions of a given area of body surface, and (2) to anaesthetize, if possible, a surface differentially. The latter method seems to me untrustworthy when used alone. A 'universal sense organ' when slightly anaesthetized might not respond to light, but might respond to other more vigorous kinds of stimuli, such as the application of an acid. If, however, one finds, in addition to differential anaesthesia, the existence of definite regions which respond, e.g., to touch alone, or chemical agents alone, then one must conclude that special sense organs are present. The same conclusion is drawn if one finds that the histological structure of the sense organs in one region differs

<sup>1</sup> In strict analysis 'distance-receptor' is a misnomer, for organs to which this is applied depend, like all other sensory organs, on actual contact with the stimulating agent. It is therefore probable that '(2)' and '(3)' are only different ways of expressing the same thing.

markedly from that of sense organs in other regions. But neither of these conditions have, as yet, been demonstrated for synaptids

In Miss Langdon's ('95, p. 215) account of the sense organs of *Lumbricus*, she estimates that in a worm 19 cm. long with 152 metameres, there are 150,000 sense organs present, about 1000 to a single metamere. The magnitude of the task of stimulating separate sense organs in the earthworm can thus be readily appreciated. In regard to the histological structure of these organs she says (p. 226), "Since these sense organs form the only known sensory apparatus of *Lumbricus*, and since their structure is not visibly different in different parts of the body, it is likely that they are sense organs of a general nature capable of reacting to mechanical, chemical, thermal or luminous stimuli." She also finds (p. 218) that "the sense organs are distributed over the entire surface of the body, but are most numerous and largest at each end." Since these features of the worm are exactly paralleled by those of *Synaptula*, it would not be unreasonable to draw the same conclusion here, i.e., that in *Synaptula* also there are present "universal sense organs."

From the foregoing account it is possible to defend the following statements regarding *Synaptula*: (1) it has a well developed chemical sense, since it responds to many of the categories of substances which are stimulating to man, viz., sour, bitter, salt, sweet (?), and alkaline; (2) the order of sensitivity in different parts of the body to chemical agents is the same as to light, and to touch; (3) except for the eyes and otocysts, only one type of sense organ has, as yet, been described for it; (4) the relative abundance of the sense organs in the three or four chief regions of the animal is the same as the order of sensitivity in these regions to these three kinds of stimuli; chemical, photic and tactile.

In connection with the idea of 'universal sense organs', it is interesting to note, and not without significance, that, as the studies of Herrick and Coghill ('15) have shown, "in the development of the nervous system of *Amphibia*, the first reflex circuits to come to maturity are made up of rather complex

chains of neurones so arranged as to permit only one type of response—viz., a total reaction (the swimming movement)—from all possible forms of stimulation, and that in successive later stages this generalized type is gradually replaced by a series of special reflexes involving more diversified movements. . . . The simple reflex arc . . . , which is adapted for the execution of a single movement in response to a particular stimulus, is the final stage in the developmental process, whose initial stages are much more complex and diffuse arrangements of neurones adapted for total reactions of a more general sort" (Herrick, '15, p. 66).

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## FOOD-REACTIONS OF PELOMYXA CAROLINENSIS WILSON

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### INTRODUCTION

All food reactions of *Pelomyxa* involve movement. Verworn, Rhumbler, Loeb and others have seen in the analogy between certain surface tension phenomena and the locomotion of Rhizopoda suggested experiments of the manner in which certain Lobosa travel. Dellinger ('06) and Jennings ('06), however, found that the movements of amoebae could not be interpreted so as to be explained in this way. Jennings ('06) says: "As our account shows, most amoebae do not move at all as do liquid drops whose movements are produced through changes in surface tension," page 5. Quite recently Mast and Root ('16) in an ingenious fashion have been able to show that surface tension is "probably at best an insignificant factor in the process of feeding in amoeba," page 48.

Wilson ('00) says: "Occasionally a *Pelomyxa* was observed in the remarkable attitude shown in figure 5, a portion of the body resting on the bottom, while the rest of the animal divided into pseudopodia projected freely upward in the water. In this condition, *Pelomyxa* appears for the time being as an attached rhizopod with tentacle-like pseudopods, the nearest analogue to which is the interesting minute form, *Hylamoeba sessilis*, found by Frensel in Cordova, in Argentina." In this connection Wilson shows in his figures variously curved regions of the body, which suggest that there might have been local torsion or bending. Such torsion was observed by Mr. Conway Zirkle in this laboratory. He saw a specimen "sway one of its longer pseudopods through an arc of nearly 90 degrees back toward the thicker region of the body. There was no perceptible flow of the endoplasm in this pseudopod."

One of us has observed an arched specimen attached at its two ends standing with the plane of the arch at right angles to the surface. This attitude has been described by Wilson ('00). But in this case, the animal swayed to and fro upon the two bases of the arch, so that at one end of the oscillation it lay with the plane of the arch horizontal and directed toward the right, then after swaying through 180°, the plane of the arch lay horizontal and directed towards the left. More remarkable than this, however, has been the behavior of the specimen which was fixed by one pseudopod such as Wilson ('00) described. This specimen, standing upon the end of its body with its free end bearing three stout pseudopods, had its free end turn so as to inscribe a circular orbit counter-clockwise. These observations indicate a differential contraction of the protoplasm, most likely the ectoplasm, which approximates muscular activity and could scarcely be explained in terms of surface tension.

There are obviously two classes of stimuli—chemical and physical—concerned in the response of rhizopoda to food. When, for example, an amoeba reacts to a desmid, that lies in the light, the stimulus arises from the oxygen and other elements or compounds given off by the plant's metabolism. On

the other hand a ciliated object may produce physical stimulation through currents set up in the water as well as causing stimulation through the products of its metabolism. Schaeffer indicates that mere physical stimuli are responded to by amoeba.

Schaeffer, ('12), says: "All sorts of particles, whether digestible or not, are eaten if properly agitated. Not only is it unnecessary for the agitated particle to lie in contact with the amoeba, but vibrations produced by a needle are likewise reacted to positively, if the needle point is one-fiftieth of a millimeter or more from the amoeba. We may be certain, therefore, that water vibrations proceeding from a definitely localized source are an efficient cause for successful feeding," page 60.

As a rule, the reactions of *Pelomyxa* to food fall into two general types. These types arise out of the non-motility or motility of the prey—the possibility of escape of the latter presenting a contingency, which to be successfully met, has led to a definite type of reaction.

Great variability of reaction to food has been observed in *Amoeba proteus* by Kepner and Taliaferro ('16), though but two classes of stimulation, concerned in the reaction of a rhizopod to food, can be recognized.

We shall record at this point the following examples of the first type of food reactions. These descriptions of *Pelomyxa*'s behavior are taken from notes which were made at the time of observation by the authors and others who happened to take turns with us at the microscope as the reactions progressed.

#### OBSERVATIONS

##### *A. Reactions to objects not capable of retreat*

November 4, 1916. A *Pelomyxa* moving in the direction of a green *Eromosphaera* encountered the spherical alga eccentrically so as to turn it counter-clock-wise (as seen under the compound microscope) through about ten degrees. The *Eromosphaera* was not moved farther than this. The tip of the *Pelomyxa*'s pseudopod next expanded to fit intimately the

contour of the algal cell. This process continued until either a cup or an over arching of the Rhizopod's protoplasm had advanced to the contour *a*—figure 1. The margin *a* then advanced to contour *b* and beyond to margins *c* and *c'* which had fused, bringing the spherical green cell within a closely fitting food vacuole. Within five minutes the *Eromosphaera* was rejected by the *Pelomyxa*.

November 13, 1916. The specimen shown here reacting to two desmids, had taken through its dorsal surface two days previously a *chilomonas* and had unsuccessfully reacted to *Paramaecium caudatum*. This *Pelomyxa* came in contact with two rounded, green desmids A and B. Each desmid was then intimately surrounded and carried into the cytoplasm (fig. 2). Within five minutes, however, B was thrown out as 1 leaving a bi-forked cytoplasmic projection from which it had been dropped. Within ten minutes A had been ejected at 2 leaving a distinct cytoplasmic pocket as shown.

In the above examples we have instances of *Pelomyxas* having ingested non-motile plants, which were giving off oxygen. These plants were sooner or later rejected; but this is not always the case as the following observation shows.

November 7, 1916. Messrs. F. L. Foster and C. Zirkle alternated with us in observing a specimen that had been in a hanging drop containing long desmids and *Euglenas*. This specimen was pushing three non-motile *Euglenas* ahead of it and gliding by a fourth with which it was in contact. One of the *Euglenas* was ingested and retained.

November 27, 1916. A specimen was travelling along in the direction of the two pseudopods 1 and 1' as indicated by the arrows (fig. 3). Pseudopod 1' came in contact with a rounded bacterial mass within which were two kinds of ciliates. The longer type of ciliate was about the size of *Loxocephalus granulatus*, but for these we had no time in which to determine the genera and species. When this contact with the mass of bacteria and of ciliates was made, a reversal of the course of pseudopod 1 occurred as indicated by arrow 2 and pseudopod 3 grew out over (above) the bacterial glœa and in this way ex-

erted enough pressure to cause one of the large ciliates to leave the glœa and swim beneath the body of Pelomyxa as indicated by the line 4. At 4-a it had escaped and a curtain of protoplasm was thrown above it, driving it back beneath the body proper of Pelomyxa, from which it finally escaped along the path leading to the head-end of line 4. In the meantime pseudopods 1' and 3 advanced around the sides and over the bacterial glœa causing it to be stretched into a bi-lobed mass, the smaller lobe being squeezed out of the constricting region between the bases of the pseudopods. This smaller mass, containing two of the larger and nine of the smaller ciliates was eventually, completely constricted and rejected. The larger mass of the glœa was for the most part ingested by the means of the pseudopods 5 and 5' (fig. 4) encircling about it as indicated by the contours 6 and 6'. As the tips of 6 and 6' approached and fused, a minute mass of glœa containing one large and two small ciliates was constricted. Two portions of the original ciliate-containing glœa were thus at 11.20 a.m. rejected and the third and largest mass was ingested. After this largest mass had been ingested it was broken into small spheroidal masses, each of which was now contained in a separate food vacuole. At 12.15 p.m. the animal was just leaving the two rejected balls of bacterial glœa and showed many food vacuoles within which were active ciliates.

Thus, in this type or reaction—reactions in response to non-motile objects of prey—there is always an intimate embrace of the prey whether oxygen or carbon dioxide be the chief factor in setting up the stimulation as shown by the above examples.

#### *B. Reactions to objects capable of retreat*

Such is not the case when Pelomyxa is reacting to motile objects of prey. Flagellates and ciliates, even though they are not at the time moving from place to place, are reacted to by Pelomyxa in a definite way. A stationary Chilomonas, for example, by the lashing of its flagella gives rise to currents in the water which become a factor in the stimulus that acts upon Pelomyxa. The following examples indicate how to this gen-

eral type of stimuli, the *Pelomyxa* reacts to meet the contingency of the possible escape of such ciliates and flagellates as set up perceptible currents in the surrounding water.

When the motile prey lies in the same plane as that in which the *Pelomyxa* is moving we have observed the following examples of reaction.

October 21, 1916. This day's observations were based upon an individual that resulted when one *Pelomyxa* had been cut into four parts. It was observed in a hanging drop of spring water in which it had been since October 19 when the original *Pelomyxa* had been divided into four pieces. It was noticed that a *Chilomonas* had made a contact at a point indicated by position 1 in figure 5. Two pseudopods were thrown out almost at right angles to the direction of locomotion (indicated by the arrow). One pseudopod was anterior to the quiet *Chilomonas*, the other was posterior to it (*a* and *a'*, fig. 5). An arching film of protoplasm appeared as these pseudopods *a* and *a'* grew and curved about the prey. Thus the prey was being surrounded laterally and above and left with only one path of retreat between the advancing tips of the pseudopods and the margin of arching protoplasmic film; the surface film of the hanging drop of water prevented its escape from below. Pseudopods *a* and *a'* advanced, their ends met and then bent in upon themselves as indicated by the dotted contours of their small tips; next, the roof of the *Chilomonas*'s enclosure was completed by the advancing protoplasmic film fusing completely with the inner margins of the two pseudopods. It was only when the enclosure had been completed that the *Chilomonas* was disturbed and began to dart excitedly about to and fro within the gradually reducing enclosed space. As all these phases of the reaction ran their course the main part of the body was flowing by the part involved in the ingestion of the prey; so that by the time the *Chilomonas* lay quieted within a vacuole of the usual size the involved mass of *Pelomyxa* body lay well at the posterior end of the body proper (fig. 5, 2).

October 21, 1916. This observation was also made upon one of the four parts into which a *Pelomyxa* had been cut on

October 19, and had been kept since then in a hanging drop of spring water. The advancing rhizopod moved by a *Cryptomonas* which lay quite remote from the 'right' side of *Pelomyxa* as indicated (fig. 6, *g*). To our surprise the blunt pseudopod of the 'right' side of the body, which, heretofore, had shown no streaming, began to form a relatively narrow outgrowth, which advanced and curved in to contour *a*, and continued, curving about the *Cryptomonas g* until it had formed a widened overarching end *b* that lay along side of the body proper. In the meantime a short pseudopod, *a'*, flowed out towards elongating pseudopod *a*, meeting it and passing by its outer margin. When *b* contour had been formed pseudopod *a'* ceased its streaming movement and later was withdrawn into the main body of the animal. Two foreign particles were held in intimate contact between two opposite streams of protoplasm as *b* passed down along *a'*. (These two particles were later rejected as non-food when the unwinding of *b* and *a* took place.) Three small, upward projecting pseudopods, *c*, *c'* and *c''* next appeared. Pseudopod *b* continued to widen as it advanced to overarch, in part, the *Cryptomonas* and resulted in the approximate contour *e-e*. The overarching protoplasm was further extended by the appearance of pseudopods *d* and *d'*. These various over-arching protoplasmic processes further expanded and had their margins fuse, so that the little green plant was now enclosed between the surface film of the drop of water below and an inverted saucer-like film of protoplasm above. Pseudopod *f* arose as the margins of *a*, *b*, *c*, *c'*, *d*, *d'*, and *e-e* were fusing but it was at once withdrawn. Little forward movement was shown by the *Pelomyxa* while this saucer-like arch of protoplasm was being formed over the *Cryptomonas*; but when the saucer was completed the animal advanced and in passing by the involved mass of protoplasm partially unwound *a* and *b* so as to free the two foreign particles that had been caught between *b* and *a'*. The *Cryptomonas* now showed great agitation and vainly sought to escape as the space within which it had been entrapped was being reduced. By the time the mass of protoplasm containing the prey had reached the

'posterior' end of the body-proper, the little plant had been quieted and lay within a vacuole whose diameter was about twice the length of the *Cryptomonas*.

October 23, 1916. This reaction also was observed on one of the individuals arising when a *Pelomyxa* had been cut into four parts on October 19. We found a *Paramaecium caudatum* (fig. 7-1), 'tickling' the 'anterior' end repeatedly by darting against the *Pelomyxa* and then retreating and again advancing to encounter the *Pelomyxa* at another part of its greatly expanded end. While we waited for a response to these recurring contacts a *Loxocephalus* came to play repeatedly against the anterior end at the position 3 in figure 7. In response to these two sets of stimuli, pseudopods *a* and *a'* arose. The second one of us was now called in to collaborate in this observation. Pseudopods *a* and *a'* advanced to contours *b* and *b'*. About this time a second *Paramaecium caudatum* entered the breach and lay in the partly enclosed space. Next *b* and *b'* met at *c* and *c'*; but there was no fusion of the ectoplasm of *c* and *c'*. The inner margins of *c* and *c'* widened to form upward-projecting films of protoplasm *d* and *d'*; film *d* elongated and passed down by the side of *d'* to widen the over-arching protoplasm along contour *e*. The ciliates now began to show signs of uneasiness and darted to and fro hitting the edges of the enclosing pseudopods which could be clearly seen to deflect the ciliates towards the surface film of the hanging drop of water.

Next there appeared two outer pseudopods *f* and *f'*, which at no time became active. Finally *g*, *h*, *i*, as broad bands of over arching cytoplasm, came up over the space enclosing the three ciliates and eventually fused with the inner margins of *d* and *e* to complete the enclosing vault of the trap—the surface film of the hanging drop forming its floor. Next the space was divided, *k* and other parts of protoplasm forming a constriction. In one secondary space a paramaecium was enveloped and in the other the second paramaecium and the *Loxocephalus* were enclosed. These secondary spaces were finally reduced greatly and in the end each paramaecium was divided into three parts—



each part entering a food-vacuole. The result was there were seven food-vacuoles in the posterior end of the paramaecium by the time it had lost the spheroidal shape it had assumed in the later stages of overcoming the ciliates.

October 26, 1916. The specimen concerned with this observation had been starved for four or five days in a hanging drop of water. Paramaecia were added to its drop of water at 2.05 p.m. These many paramaecia, striking it on all sides at frequent intervals, caused it to respond at the points of contact by throwing out frequent short projections. Within five minutes, however, a paramaecium entered a bay between three pseudopods, two of which lay within the plane of the paramaecium's axis and the third one lay beneath the paramaecium. The paramaecium backed in and out of the bay at first; but as it became quieter, pseudopods *a* and *a'*, figure 8, were formed. Then *a* and *a'* widened to contour *b* and *b'*. In the meantime an over-arching film of protoplasm arose, its advance being indicated by contours 1 and 2. After this over-arching had been completed contours *b* and *b'* advanced to *c* and *c'*. We could not see what modification of the lower pseudopod took place. Eventually, however, the paramaecium was enclosed within a vacuole so small that the ciliate being bent upon itself formed a spheroidal mass. The living paramaecium, within its intimately fitting gastric vacuole was delivered to the 'posterior' end of *Pelomyxa*'s body. An interesting departure from the usual method of dealing with paramaecium was encountered in this case. Usually the paramaecium is killed as indicated by its change of form or by its fragmentation within fifteen minutes. This animal was enclosed within a rounded, intimately fitting gastric vacuole at 2.30 p.m. or earlier and within this it continued to spin periodically until 4.43 p.m. at which time its own contractile vacuoles were vigorously pulsating. We hope to make this type of variability the subject of another study.

The following observation indicated the manner in which the contingencies of the situation are met when the motile object of prey lies above the plane in which *Pelomyxa* is traveling.

November 3, 1916. Pseudopod *a* moved beneath a quiet *Chilomonas* until it had travelled to *a'*, figure 9. The pseudopod *a'* now expanded beneath the *Chilomonas* to the contour *b-b'*. A cup was evidently formed about the flagellate, though the details of this cup's formation could not be seen; for eventually the *Chilomonas* began struggling within a space about as large as was the diameter of pseudopod *a'*. The space became more and more reduced as the *Pelomyxa* flowed on, until eventually the *Chilomonas* lay quiet within a small vacuole at the posterior end of the animal.

The details of *Pelomyxa*'s reaction to a quiet, motile body, lying beneath the plane of its movement, seem to be the reverse of those made with reference to an animal that lies above the plane along which the *Pelomyxa* is moving.

November 15, 1916. A small specimen displayed currents in the two pseudopods indicated in figure 10. The shorter one of these pseudopods traveled over a quiet paramaecium until its end lay at *a*. This narrow pseudopod then expanded to contour *b-b'*. The margins of this expanded pseudopod supported a somewhat circular curtain that was being dropped about the paramaecium. Thus an inverted cup was being formed about the paramaecium, which had been quiet except for the action of its peristomal cilia. While this was taking place, the paramaecium escaped and the modified pseudopod was withdrawn without the completion of the cup.

Perhaps the most interesting manner of meeting the contingency presented by the possible escape of the prey is given in the following reaction.

November 11, 1916. A *Chilomonas* was playing in the bay formed between one side of *Pelomyxa*'s body and a desmid that lay in contact with the rhizopod. In response to this but one pseudopod was sent out which advanced to make a contact with the desmid (*a*, fig. 11). The direction of growth was changed in this pseudopod after it came in contact with the outer segment of the desmid so that its advance was in towards the body-proper along the side of the desmid (*b* and *c*, fig. 11). In the meantime the *Chilomonas* was enclosed at its second

position (2, fig. 11). Protoplasm overarched the somewhat triangular bay and eventually the specimen was crowded into a relatively small food vacuole and quieted.

Reactions once started may or may not be reversed when the prey escapes.

October 23, 1916. This observation was based upon one of the four parts into which an individual had been divided on October 19. In this case a small, very active ciliate (about the size of *Chilomonas*) entered the angle between the two anterior pseudopods A and B, figure 12. This ciliate glided back and forth along the middle third of the mesial surface of pseudopod A. This pseudopod widened to contour *a-a* and *a'-a'* and then small pseudopods *b* and *b'* arose one to each side of the persistently gyrating ciliate, while a similar pseudopod *c* arose from the mesial surface of B. The ciliate, however, escaped at this point along the path indicated by arrow *y*. Pseudopods *b*, *b'*, and *c* were then retracted and pseudopod A narrowed.

November 13, 1916. A specimen of *Pelomyxa*, that had in its posterior end an ingested paramaecium which was pulled into a bilobed contour, approached along its main axis a group of three paramaecia. The anterior end became bifurcated before a contact had been made with the ciliates. This bifurcation continued resulting in the formation of pseudopods *a* and *a'*, fig. 13 A. The remotest of the three paramaecia retreated before the reaction had well set in. Pseudopods *a* and *a'* advanced until the latter collided with the nearest paramaecium and drove it away. The third paramaecium was disturbed and chased away by pseudopod *b'* hitting it. There were now no objects of prey within the enclosed bay and yet *b* bent upon itself in its advance to *c*, while *c'* crossed in beneath *c* as a prolongation of *b'*. Furthermore a thin overarching pseudopod *d* arose from the fundus of the bay and as it expanded, to meet the expanding, overarching margins of *a*, *a'*, *b*, *b'* and *c'* the completion of a well formed food vacuole was effected.

This same specimen a little later had a Paramaecium strike its anterior end to which it responded by sending pseudopods

*a* and *a'* (fig. 13 B) about the ciliate. The prey now escaped, but *a'* was advanced to *b* and an overarching thin pseudopod *c* arose from between the bases of the two encircling pseudopods. The formation of the food vacuole did not advance beyond this state. Pseudopods *a*, *b-a'* and *c* were then retracted.

*Pelomyxa* may also modify its reaction to a given stimulus as though a summation of stimuli, or a change of physiological state had taken place. This is shown in the following observation in which the *Pelomyxa* was reacting to a *Paramecium caudatum*.

November 14, 1916. The *Pelomyxa* in this case moved past a quiet *paramecium*, which lay almost parallel to and with its oral or ventral side directed away from the rhizopod. After the *Pelomyxa*'s anterior half had passed the ciliate it reacted by sending a small pseudopod *a* (fig. 14) to the *paramecium* at position 1. The contact with the *paramecium* caused it to take position 2. The narrow pseudopod followed the ciliate and made a second contact at *b*. Again the *paramecium* retreated. It was followed to its third position 3 and contact *c* was made. *Paramecium* next took position 4. The tapering pseudopod followed it to *d* and again caused it to move off; this time to position 5. At this point the advancing pseudopod, *a*, *b*, *c*, *d*, was modified so that it grew laterally as it advanced towards position 5 of the *paramecium*, and when it came close to this animal it spread anteriorly and posteriorly along the dorsal side of the prey. In the meantime the entire pseudopod widened as the anterior portion of the *Pelomyxa*'s body flowed back into it. This widened pseudopod (shown by the broken contours) now gave rise to pseudopods *f* and *f'*, these in turn advanced to *g* and *g'* when the *paramecium* escaped from the mouth of the enclosed bay. After this the large bifurcated pseudopod was slowly withdrawn.

## DISCUSSION

The reaction to food on the part of *Pelomyxa* involves primarily a differential contractility. The ability to exercise local contraction of the body is displayed by the observation of H. V. Wilson ('00), Mr. Zirkle and one of us. Such conduct could not be expected to be explained through surface tension phenomena and brings to bear upon the problem of food-vacuole formation evidence somewhat of the sort that Mast and Root ('16) so ingeniously derived from the reaction of *Amoeba proteus* to paramaecia.

Moreover, the formation of food-vacuoles does not involve adhesion as Bayliss ('15) suggests in the following: when the surface of an amoeba or other rhizopod "comes into contact with food, it appears to soften and become sticky so that the food adheres and is more readily taken in," page 22. No evidence of adhesion has been encountered in either type of food reaction of *Pelomyxa*.

Schaeffer ('16), in some of his observations on amoeba came upon reactions closely resembling the two general types we have been able to record above. He says "a small organism like the flagellate *Chilomonas* is ingested in a food-cup nearly always large enough to accommodate easily the ciliate *Coleps*. But particles of isolated proteins are frequently ingested in food-cups scarcely larger than the particles themselves," page 545, "and actively moving objects such as organisms, and mechanically agitated particles are eaten with more water than quiet objects," page 547. If this were all that he was able to record concerning the amoeba's reaction to moving and non-moving objects, his records would closely agree with our observations of *Pelomyxa*'s two types of reactions to food. He goes on to say, however, that "Some objects such as isolated proteins are sometimes ingested apparently without any water; sometimes with just a slight amount of water. On other occasions again, the same kind of objects are ingested in food cups with larger amounts of water," page 547. In *Pelomyxa* we have found no such variability. When non-motile food was ingested there

was always the smallest possible amount of water involved in the formation of the food-vacuole.

Schaeffer ('12) says: "Encircling seems to take place whenever the stimuli coming from a particle are not sufficiently strong to produce movement directly toward the source of the stimulus, nor weak enough to be ignored by the amoeba. But these conditions would not be sufficient to produce encircling such as we see in amoeba. Such conditions would doubtless produce uncertain behavior, but it would not necessarily express itself in encircling the test object. There must, therefore, be another factor present in amoeba which is concerned with encircling. This factor is a tendency to continue moving forward after movement is once started. The amoeba seems to acquire some sort of a momentum of reaction, which tends to keep amoeba moving in more or less straight paths. Such a tendency balanced against stimuli producing mild positive behavior would result in encircling movements as illustrated in figures 2 and 3," page 63-64.

It appears to us that an effort to apply physiological momentum as a factor to explain encircling movement in *Pelomyxa* would be futile. Because we have frequently seen a food-vacuole well advanced in its formation, which after the prey had escaped was reversed. Figure 13, A and B shows two reactions in which the encircling movement cannot be explained by physiological momentum. When reaction A set in, there were three paramaecia involved. Before the reaction had well begun the remote paramaecium retreated. When pseudopods *a* and *a'* had been formed the latter striking the nearest ciliate, caused it to retreat leaving only the middle paramaecium. The withdrawal of the remoter paramaecium, no doubt, but little modified the intensity of stimulus; but when the nearer ciliate retreated the strongest source of stimulation must have been removed. It was interesting, therefore, to note that though the stimulus was weakened, the pseudopods continued to diverge as they grew. Moreover, when pseudopod *b'* hit the remaining paramaecium and caused it to retreat the further reduction of the stimulation to zero resulted no longer in a divergence, but in

a convergence of the growing pseudopod. Here food-vacuoles were thus formed in a complex fashion despite the fact that the reaction had advanced to form only *b* and *b'* when the ciliates had all escaped. This might suggest a strong physiological momentum causing, by a flood-like process, the completion of the encircling movements. However, but a little later in the same forenoon this same animal had almost completed the encircling movement about a paramecium indicated at figure 13, B-*a* and *b*, and had also formed an overarching pseudopod *c* over the enclosed bay before the paramecium escaped. The entire reaction, therefore, in this second instance was much more advanced than in the first (fig. 13, A), and yet after the escape of the prey the currents in pseudopods *a*, *b*, *c*, figure 13, B were reversed at once and the pseudopods withdrawn.

In the second place, there is yet another method of surrounding an animal that can hardly have the conception of physiological momentum applied to it. Kepner and Taliaferro ('16) saw that in *Amoeba proteus*, when a *Chilomonas* stimulated the rhizopod in the narrow angle between two pseudopods that lay close to each other, a small pseudopod was given off behind the *Chilomonas* from the mesial side of each large pseudopod. We have seen something somewhat like this in *Pelomyxa*. In figure 12 there is shown a reaction to an object that was stimulating the mesial side of one of two adjacent pseudopods (A and B). Here, pseudopod A, after it had by expanding mesially, crowded the small ciliate over closer to pseudopod B, threw out to each side of the possible prey a small secondary pseudopod; while pseudopod B threw out a secondary pseudopod *c* toward *b* of A. Before the secondary pseudopods *b* and *c* had closed to cut off the ciliate's retreat the latter escaped along the path *y*. After this escape the entire reaction of *Pelomyxa* was reversed. Here we have a greatly agitated body causing an encircling movement. More than that, after the escape of the prey, though the reaction was well advanced, it was reversed at once. The sudden reversal of relations, more advanced than others which completed the formation of food-vacuoles, suggests that physiological momentum can hardly be invoked to explain the reaction of *Pelomyxa* towards motile food.

Starved *Pelomyxa*s and those containing food show a constancy in the rate of reaction towards the two types of food motile and non-motile). We have not had it indicated that "differences in the intensity of hunger determine, at least to some extent, whether the globulin shall be eaten by means of a food-cup or not, and whether the quantity of water taken in with the globulin shall be large or small," Schaeffer ('17), page 64.

In the majority of cases of encircling reactions to food, it would appear that conditions would impose a gradual increase in the intensity of stimulation; for as a *Pelomyxa*'s encircling pseudopods close about a paramaecium, the disturbance of the enclosed water by the cilia of the protozoan must be increased; and, in addition, the concentration of carbon dioxide and other excretions of the ciliate must become more (pronounced) thereby causing a greatly intensified stimulation. This increase in intensity of stimulation does not lead to an acceleration of the rate of reaction. Verworn ('13) says: "All phenomena can change in their rapidity as well as in their nature. That is, quantitatively and qualitatively. In this way the specific vital process of an organism can be altered by the stimulus, on the one hand, in its rapidity; on the other, in the manner of its reaction. The majority of temporary responses to stimuli consist in alterations of rapidity of the vital process, and from either a quickening or retardation of its course," page 73. In the majority of encircling movements, in which there is an increase in the stimulation, we do not have a 'quickening' nor a 'retardation' of the course of the reactions. Again, in examples in which objects of prey escape in a manner that by irregular gradation reduces the amount of stimulation, the reaction may not be accelerated, though sustained until the completion of food-vacuoles (fig. 13, A); while in other instances the removal of stimulation results in a reversal of the reaction (fig. 13, B and fig. 12). The reaction of *Pelomyxa* to motile objects of prey is, therefore, primarily a qualitative one.

Because of the qualitative character of the reactions of *Pelomyxa* we cannot recognize in them factors that can be reduced



to terms of equilibration between the organism and its environment, or in the words of Lillie ('15): "In any stable or well-adapted species the total action of the environment upon the organism is exactly counterbalanced by the total activities of the organism. The interaction is reciprocal, and normally results in an adjustment which renders prolonged equilibrium possible. . . . Each organic feature has its complement in some feature of the environment. The complexity of the organism is thus the correlative or mirror-image of the complexity of external nature." Our own work, on the other hand, indicates that a striking characteristic of the food-reactions of *Pelomyxa* is their adaptability to varying present conditions which involve contingencies. Lillie says also: "It is these externally directed actions which form the greater part of what is known as 'animal behavior,' and they represent an important though not the only, means by which the animal adapts itself to its environment.

"Ordinarily we class such actions partly as 'instinctive' partly as 'intelligent'; perhaps their most remarkable feature is that they often have reference to a future more or less remote—they can not be understood by taking to account present conditions alone." The contingency of escape of the possible prey contrasted with no such contingency are factors in differentiating the two general types of reaction. Moreover, the relative position of the animal that is likely to escape presents conditions that are met in a highly variable manner. For example: (1) When the motile prey lies below the *Pelomyxa*, a curtain of protoplasm is dropped down about the prey and then eventually the margin of the curtain closes beneath it to complete the formation of the food-vacuole: (2) When the motile prey lies above the rhizopod a cylindrical cup arises about it, the lips of this cup eventually closing above the captured animal: (3) When the motile prey lies in the plane through which the *Pelomyxa* is advancing pseudopods are first sent out to each side of the prey and when it is quite surrounded in this plane, overarching pseudopods form and finally the protoplasm closes in beneath it: (4) If the prey lies between pseudopods it may

be crowded towards one of the pseudopods by the expansion of one of them until the inter-pseudopodial space is reduced when the two pseudopods take part in the formation of the food-vacuole; and (5) finally, even a foreign body, such as a desmid may become a conditioning factor in the reaction of the *Pelomyxa* in meeting the contingency of the escape of the prey.

This variability of reaction to food of *Pelomyxa* stands in sharp contrast to that of a more highly specialized protozoan such as *paramaecium*. Similarly there is a contrast between types of food eaten by the two animals. A *Pelomyxa*'s food varies in quality greatly, for it includes plants and animals; in size this food varies from nematodes to very minute ciliates and flagellates. A *paramaecium*'s food, on the other hand, consists solely of bacteria and its size is determined by the carrying capacity of the vortex created by its peristomial cilia. Hence we find *Pelomyxa* having two types of food-reaction and presenting great variation in the details of one of these; whereas, *paramaecium* has but one type of food reaction and that showing little if any variation.

#### SUMMARY

1. There are two general types of food-reaction.
2. The contingency presented by the possible escape of motile objects of prey appears to condition the more complex type of food-reaction.
3. The paths of possible retreat of motile objects present a wide range of variability, so that, though we may predict what in general the type of reaction may be to such objects, yet the details of such reactions cannot be predicted. Slight variability of contingent conditions is presented by non-motile objects of food, and to these we find *Pelomyxa* responding with very little variability of reaction.
4. No hypothesis, yet advanced to explain the movement of Rhizopoda, can be applied to the solution of the problem of *Pelomyxa*'s movement about the food-bodies.

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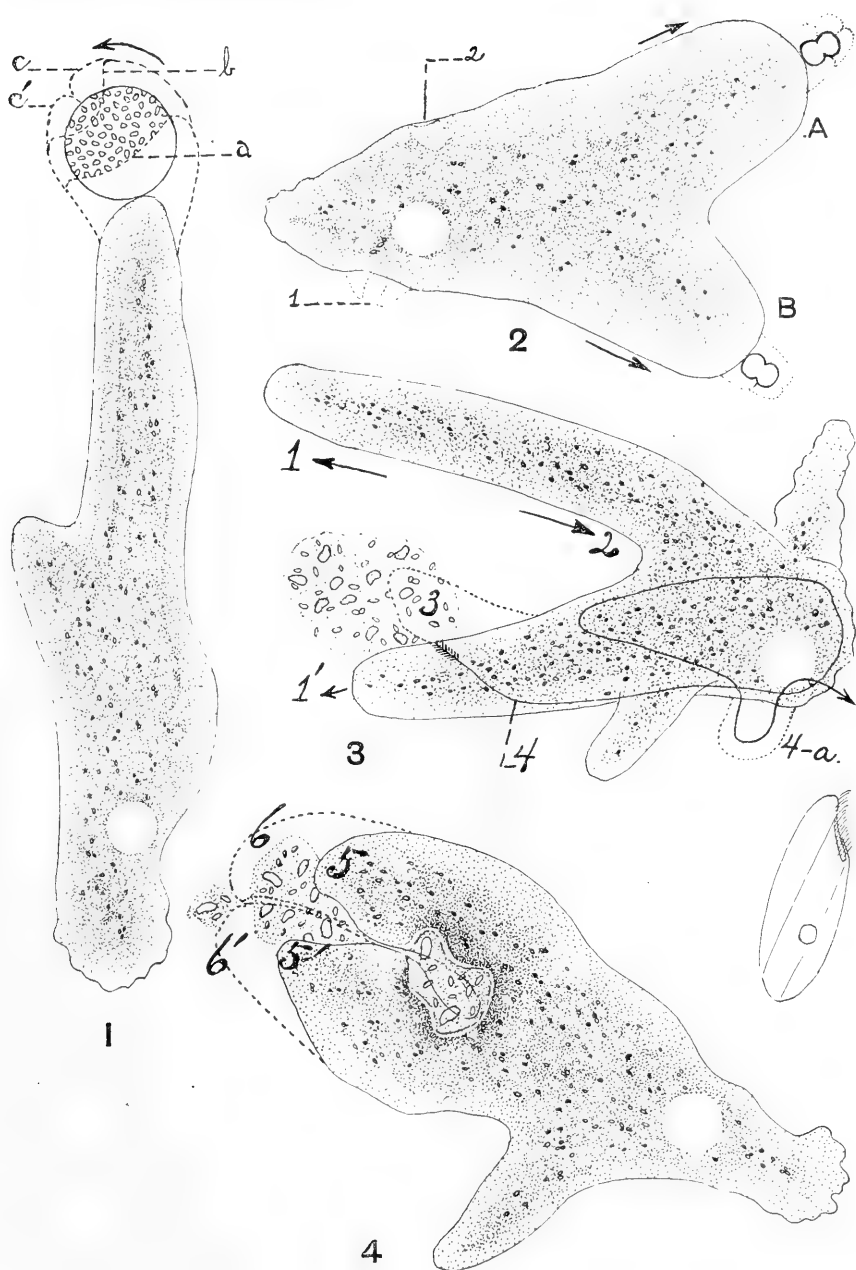
## PLATE 1

### EXPLANATION OF FIGURES

1 A specimen encountering a green plant, *Eromosphaera*. The arrow indicates the direction in which the spherical plant-cell was turned when the *Pelomyxa* first came in contact with it.  $\times 100$ .

2 *Pelomyxa* reacting to two desmids, which had been encountered synchronously. The desmid at pseudopod B was rejected at 1; that at pseudopod A was rejected at 2.  $\times 100$ .

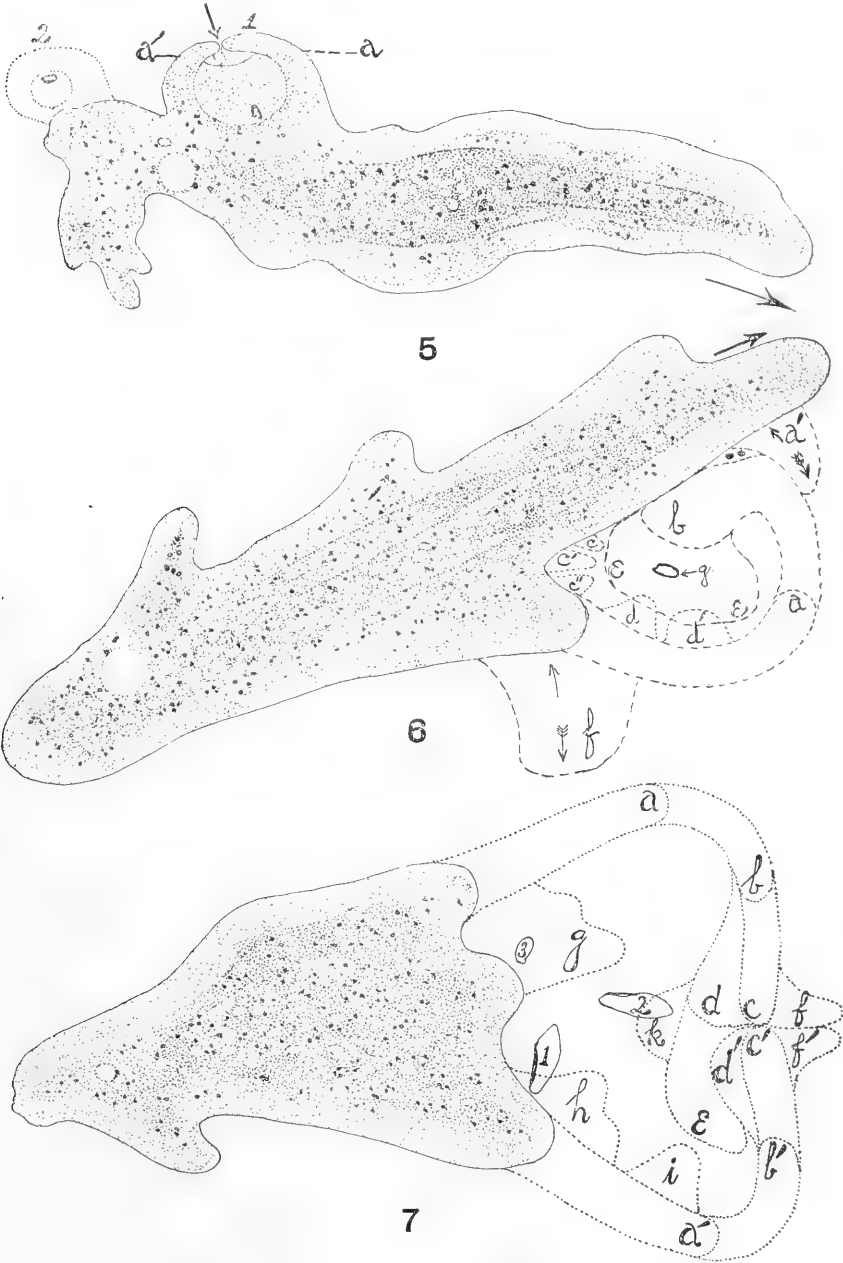
3 and 4 A specimen reacting to a non-motile ciliate-containing glea of bacteria. The outline to the right shows the contour of the larger kind of ciliate that was enclosed in the glea.  $\times 100$ .



## PLATE 2

### EXPLANATION OF FIGURES

- 5 *Pelomyxa* reacting to *Chilomonas* which had made contact with it as it came along path indicated by arrow.  $\times 100$ .
- 6 *Pelomyxa* reacting to a *Cryptomonas* with which it had not come in contact.  $\times 100$ .
- 7 *Polomyxa* reacting to two *paramecia* and a smaller ciliate.  $\times 100$ .



### PLATE 3

#### EXPLANATION OF FIGURES

8 *Pelomyxa* reacting to a paramaecium that lies above one of its pseudopods and between two other pseudopods.  $\times 100$ .

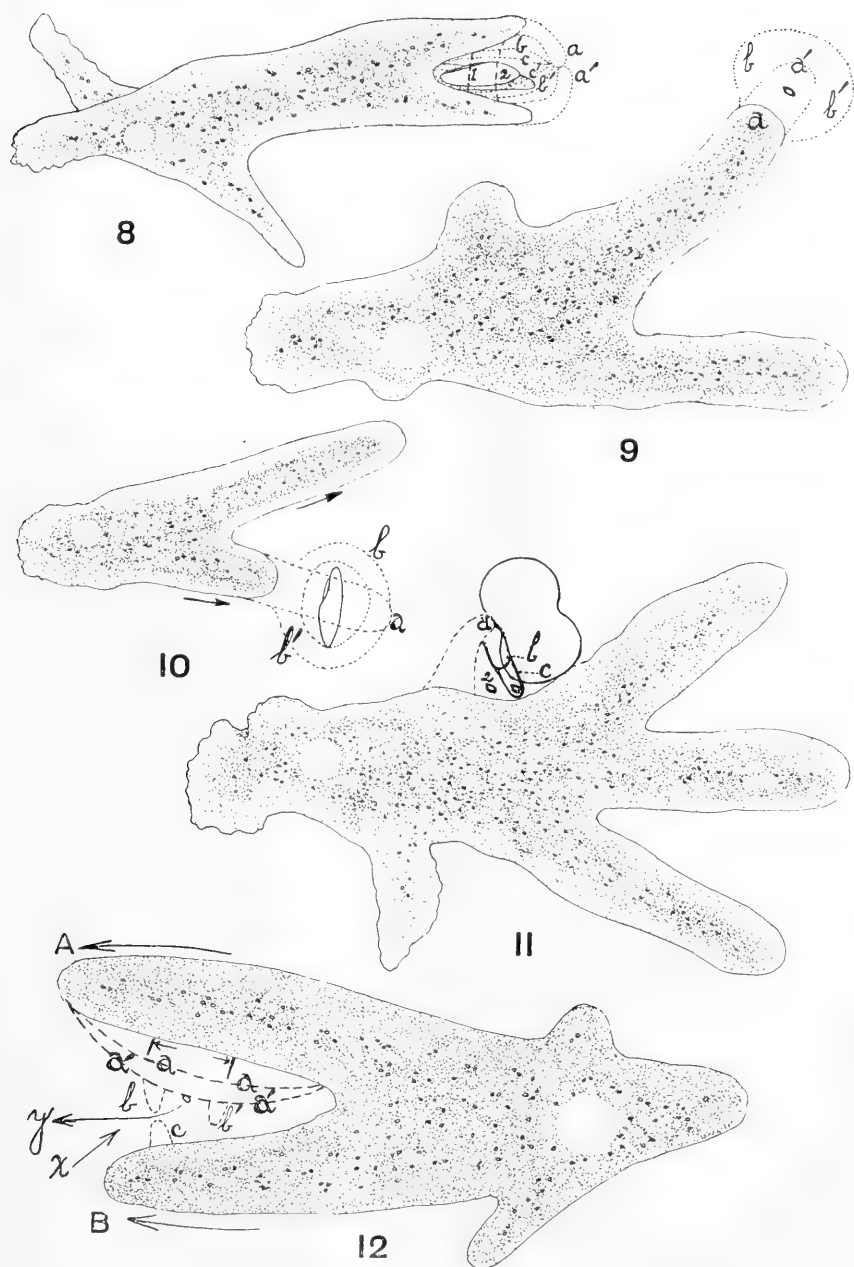
9 *Pelomyxa* reacting to a *Chilomonas* that lies above the plane in which the rhizopod is lying.  $\times 100$ .

10 *Pelomyxa* reacting to a paramaecium that lies below the plane in which the rhizopod is lying.  $\times 100$ .

11 *Pelomyxa* capturing a *Chilomonas* that lies in the angle between the body-proper of the rhizopod and a desmid. Pseudopod *a*, when it came in contact with the desmid, was deflected to grow to *b* and then to *c*.  $\times 100$ .

12 *Pelomyxa* reacting to a small ciliate, that, coming along path shown by arrow *x*, played to and fro against the inner side of pseudopod *A* between the two lines against which small arrow heads lie. As the pseudopod was thus being stimulated it widened to contour *a-a* and then to contour *a'-a'*. From this latter contour two small pseudopods, *b* and *b'* were thrown out towards pseudopod *B* and small pseudopod *c* arose from the mesial side of *B* opposite pseudopod *b*. At this point the prey escaped along the path *y*.  $\times 100$ .





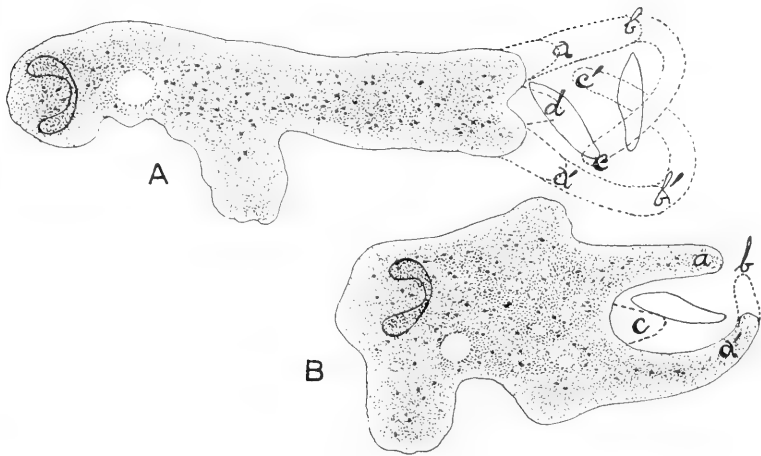
## PLATE 4

### EXPLANATION OF FIGURES

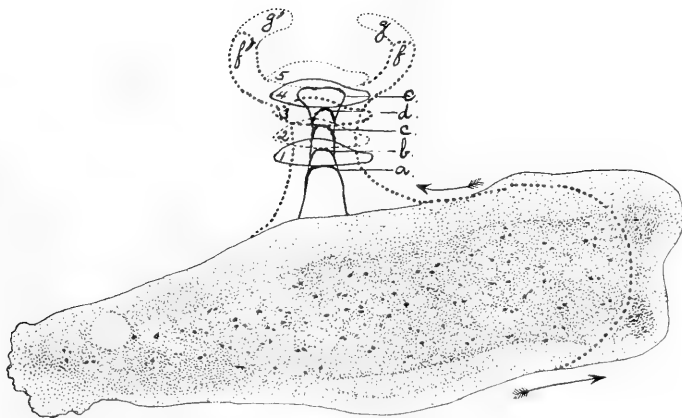
13 A. *Pelomyxa* reacting to a group of paramaecia. All of these ciliates escaped by the time pseudopods *b* and *b'* had formed. In this case a food-vacuole was completed.

B. The same *pelomyxa* reacting to a paramaecium, which escaped when pseudopods *a b-a'*, and *c* were formed. After the escape of the prey, the pseudopods *a b-a'*, and *c* were retracted.  $\times 100$ .

14 *Pelomyxa* reacting to paramaecium four times in a similar manner. After this, its fifth reaction was changed by having the tapering pseudopodium expand at the end and thus embrace the ciliate by pseudopods *g* and *g'*.  $\times 100$ .



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## EXPERIMENTAL STUDIES ON THE ORIGIN OF MONSTERS<sup>1</sup>

### II. REGARDING THE MORPHOGENESIS OF DUPLICITIES<sup>2</sup>

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TWENTY-SEVEN FIGURES

#### INTRODUCTION

In a recent publication (Werber, '16 b) I have attempted an etiology of monstrous development and an analysis of the morphogenetic factors underlying it. The observations there recorded and the theoretical conclusions based on them pertained largely to terata of the head, but some other experimentally produced forms of pathological development have also been sufficiently considered.<sup>3</sup>

The present paper is a continuation of this work. Its primary purpose is to put on record some duplicities which have resulted from the employment of a chemical method. The attempt is also made to account for their morphogenesis although I am fully aware of certain possible inadequacies of our

<sup>1</sup> This contribution is a part of the work carried on with the aid of a grant from the Bache Fund of the National Academy of Sciences in 1915.

<sup>2</sup> The term 'duplicity' has, unfortunately, lost its original meaning and come to be employed almost exclusively in its metaphoric sense. In view of the lack of an other suitable English term I propose henceforth in dealing with monozygotic double embryos to employ it in its original meaning which I believe to be proper and precise, being the equivalent of the Latin 'duplicitas' and the German 'Doppelbildung.' The commonly employed term 'double monsters' ('Doppelmissbildung') can be employed only for deformed conjoined double embryos.

<sup>3</sup> In a very recent paper Newman (Biological Bulletin, vol 32, No. 5, 1917) makes the statement that I failed to account for such teratomata as the 'isolated eye' (or the 'solitary eye'). In reply I refer the reader to pp. 541-552 of my 1916 b paper where these teratomata, for the first time experimentally produced by myself, are described and their morphogenesis fully accounted for.

interpretation which may be largely due to the insufficiency of data at command at the present time.

I also appreciate the justification of an objection often raised against such attempts, namely that deductions regarding the morphogenesis of monsters are uncertain, if based only on the analysis of the end-products of such atypical development.<sup>4</sup> It is, of course, obvious that certainty could here be gained only by direct observation in vivo from the beginning of development through successive stages. However, this direct method presents great difficulties in view of which it seems necessary to take into account the inferential clues often furnished by the analysis of already developed monsters.<sup>5</sup> The indirect evidence thus gained is in accord with data established by direct observation of successive stages during atypical development in many experiments in the lower animals.

#### BLASTOLYSIS AS A MORPHOGENETIC FACTOR IN THE DEVELOPMENT OF DOUBLE MONSTERS

While attempting to account for the morphogenesis of 'monstra per defectum' I (l.c.) have shown that in many of the embryos of which a microscopical study was made there was unquestionable evidence of either dissociation of tissues or of dislocation of organs or of both. From these observations<sup>6</sup> the conclusion was drawn that we are here, manifestly, confronted with some action (or actions) that tended to disintegrate and to dissociate parts of the earliest embryonic primordium. As the chief components of this complex process (blastolysis)

<sup>4</sup> These objections were pointed out already by Rauber ('79-'80).

<sup>5</sup> Every teratological experiment in which, like the present, a chemical modification of the environment is employed, must be carried out on a great many eggs, since their mortality is very high. This greatly increases the difficulty of continuous observation of the still surviving eggs of which probably no two develop alike. Besides, the chemical processes and the alterations they are responsible for, elude direct observation. This is particularly true for the translucent egg of *Fundulus*.

<sup>6</sup> Similar observations were made by Mall ('08) who, however, dealing with material not coming from experiments, did not attempt to account for the factors underlying this shifting and dissociation of tissues which he correctly pronounced as 'histolysis.'

I regard destruction of parts of the primordium by chemical action and fragmentation resulting from the latter as well as from differences between the osmotic pressure in the eggs and that of their surrounding, experimentally modified, medium.

These conclusions can, I think, be extended to the morphogenesis of 'monstra per excessum.' For Driesch ('93) has demonstrated for the sea-urchin and Wilson ('93) for Branchiostoma that the development of various 'monozygotie'<sup>7</sup> double or multiple embryos can be induced by the employment during the first or the second cleavage of a dissociating force such as raising the temperature or shaking the eggs, which would separate the blastomeres. Similar results were obtained also by Fischel ('98) by mechanical pressure exerted on the eggs of the ctenophore *Beroë ovata*. It was further shown by Loeb ('95) that like results can be obtained in sea-urchin eggs transferred soon after fertilization to a medium of lowered osmotic pressure.

Of particular interest for our consideration is also the work of Bataillon ('01) who produced duplicities experimentally in lower vertebrates (*Petromyzon* and the teleost *Leuciscus rutilus*) by increasing the osmotic pressure of the surrounding medium. Employing this method Bataillon was able to ascertain by direct observation in *Petromyzon* that the development of monozygotie twins and various other duplicities resulted from the more or less complete separation of the blastomeres of the two-celled stage. He also states that in *Petromyzon* at least one-third of the egg seems to be necessary for the development of a whole embryo.

This equi- and totipotency of the first blastomeres was demonstrated also in the amphibians. Thus O. Schultze ('94) and Wetzel ('95 and '96) have succeeded in producing conjoined double embryos in *Rana fusca* by inverting for some length of time eggs in the two-celled stage, previously compressed between two glass plates.

Even complete monozygotie twins (of half the normal size may be obtained in amphibians by separating the blastomeres, as shown by the ingenious experiments of Herlitzka ('95 and

<sup>7</sup> This very precise term is adopted from Newman ('17).

'97) on the eggs of *Triton cristatus*. These interesting results were subsequently supplemented by the important experiments of Spemann ('00-'04) who produced various duplicities in *Triton taeniatus* by incomplete constriction of the eggs along the first cleavage plane or along the corresponding plane at any stage up to the gastrula.

Bearing in mind these data as well as also Morgan's much earlier ('93) discovery of the totipotency of the blastomeres after the first cleavage in the teleost egg (*Fundulus*) it would seem perfectly safe to assume that the duplicities recorded in our experiments (on the eggs of the same species) have resulted from (primarily osmotic) dissociation ('blastotomy'—Bataillon, l.c.).<sup>8</sup>

The origin of the double embryonic anlage can, in this way, be accounted for without difficulty—at least in anamniotes. So far as this point alone is concerned it would seem no longer necessary to resort to such assumptions as 'dichotomous growth' at the anterior end of the embryonic anlage (Gerlach, '82), or the oft-assumed binucleate eggs and coincidental polyspermy as the agent causing duplication, or the 'radiation'-theory of Rauber ('79-80) and its modified offshoot, the 'double gastrulation'-theory of O. Hertwig ('92, '06).

The inadequacies of all these hypotheses have been clearly pointed out by Fischel ('02) and in full agreement with the latter also by Schwalbe ('07). To Fischel we also owe what may be regarded as the most rational analysis of the manner in which the various combinations of duplicities in teleosts may be formed.

Fischel's considerations are based primarily on the assumption of a duplication of the germinal anlage soon after fertilization probably by abnormal osmotic pressure, as demonstrated by Bataillon (l.c.). Starting thus with two embryonic primordia and bearing in mind the mode of formation of the embryo as established (particularly for the teleosts) by several authors and notably by Kopsch ('96) Fischel, by employing the dia-

<sup>8</sup> This assumption might reasonably be extended to the double embryos often recorded in fish hatcheries (notably in *Salmonidae*), where an abnormal osmotic pressure may be due to temporary impurities of the water.



grams of the latter demonstrates with almost mathematical precision how the development of any diplopagus may result from the variation in the degree of approximation and the angle of incline of two embryonic primordia.<sup>9</sup> It is not practicable at this place adequately to present Fischel's views. They have, however, been fully discussed by Schwalbe (l.c.) to whom as well as to Fischel's paper the reader must be referred. Here it may suffice to say that his analysis accounts successfully for the morphogenesis of all double monsters, some of which (like the diprosopos, the dicephalos, the catadidymus and the meso-catadidymus) have eluded all other attempts at interpretation.

However, it must be admitted that absolute proof for all of Fischel's postulates is wanting for vertebrates higher than *Petromyzon*, since Bataillon, unfortunately, made no observations regarding the morphogenesis of the double embryos he produced by osmotic pressure in *Leuciscus*. Nor is such proof furnished by the experiments on amphibians (referred to above), as they demonstrate only how certain duplicities may result from the separation of equipotent parts of the egg, but give us no clue regarding the formation of various other duplicities (in other vertebrates) by secondary fusion of duplicated primordia, as assumed by Fischel.

It may also be mentioned, in passing, that Fischel's theory (blastotomy by osmotic pressure and subsequent chance configuration of the duplicated primordia) does not account for the malformations often exhibited by duplicities. The point is not an unimportant one, for as I shall attempt to show, these deformities may often be syngenetic with the doubling of the primordium. Besides, as will be pointed out later, the deformities of one or both components of a duplicity give us an important clue to the morphogenesis of the 'parasitic double monsters.'

<sup>9</sup> The various possible combinations in man were portrayed very suggestively in the well known diagrams of Wilder ('04) who, however, in his more recent work ('08) has abandoned his former views on diplogenesi in favor of his present 'theory of Cosmobia.'

The deformities observed in duplicities are of the same nature as those of other monsters, i.e., they are, usually, due to some defects and, sometimes,<sup>10</sup> to an inhibition in development.

It is relatively easy to account for the genesis of defects as the result of lesions produced by osmotic pressure.<sup>11</sup> The latter, however, will not account for an inhibition of development such as may be found in some monsters and, occasionally, in one or both components of a duplicity. As I have pointed out elsewhere (Werber, '15 and 16 b) for this form of deviation from the ontogenetic norm we must assume chemical alteration as responsible for the decrease of the germ's inherent capacity for development and differentiation. This assumption will account for such deformities observed in duplicities as may properly be regarded as due to an inhibition.<sup>12</sup>

Moreover, chemical action may play a much greater part in contributing to the genesis of defects by either directly destroying certain parts of the germ or by lowering their resistance to the action of osmotic pressure. The combined action of these factors (osmotic pressure and chemical alteration) may result in any conceivable monstrosity depending entirely upon its degree and upon the part of the germ which has suffered most from it.<sup>13</sup>

Keeping this in mind we shall be able to understand how not only (variously malformed) duplicities may result from the

<sup>10</sup> Rarely.

<sup>11</sup> This assumption is justified in view of the effects of osmotic pressure directly observed by Loeb (*l. c.*) in the sea-urchin. It should also be noted that Loeb's method—decrease of external osmotic pressure by diluting the sea-water—excludes altogether the possibility of chemical action.

<sup>12</sup> In previous papers I ('15 and '16 b) have defined inhibition as a decrease of the germ's originally inherent 'chemomorphic' potentiality. This conception is based on the exceedingly suggestive hypothesis advanced in the last two or three decades by a number of biochemical authors and most recently presented in a very succinct and attractive form by Reichert ('14), according to which the development of an organism is a complex series of reactions in a stereochemical system. Reichert's paper came to my attention through the kindness of Prof. H. V. Wilson after the completion of the manuscript for the present paper.

<sup>13</sup> The theory of blastolysis was presented rather exhaustively in a former paper (Werber '16 b). Owing, however, to misrepresentations which it suffered at the hands of certain authors the reader will, I hope, find that a repetition of its salient points in this paper is justified.

separation of blastomeres, but also how such monsters may come about through dissociation at a later stage (up to the time of gastrulation) as in the experiments of Spemann ('00-'04) where such dissociation was effected by mechanical constriction along the potential embryo's longitudinal axis. It is only necessary to imagine that a chemical lesion of a moderate degree is sustained more or less along the germ's chief axis. Along this chemically altered area a rupture may take place owing to least resistance to osmotic pressure. Depending upon the extent of this passive 'fission,' a diprosopos, dicephalos or any diplopagus may result from the further development of the, thus partially, doubled primordium. The assumption of this chemical action, made already by Bataillon (l.c.),<sup>14</sup> does not necessarily apply to all duplicities of 'Nature' and not even to all double embryos in teleosts, for as I have already mentioned, the duplication of the embryonic anlage at any stage before the conclusion of gastrulation might be accomplished by the physical action of osmotic pressure only. And even the malformations of the components of the resulting duplicities might well be due to this factor alone.<sup>15</sup>

In my experiments, however, substances have been added to the sea water which, owing to their chemical properties are toxic (i.e., injurious to the most important life processes), and which also, owing to their molecular weights have changed the tonicity of the sea-water. It is therefore not surprising

<sup>14</sup> Bataillon (l.c.) also assumes that in his experiments chemical alteration may have played a considerable part in the genesis of defects.

<sup>15</sup> As an example I am inclined to regard the frequently observed Salmonid double monsters. In a considerable number of trout double embryos in my possession (collected in a fish hatchery in the vicinity of Freiburg i. B.) examination of sections fails to disclose anything that would suggest chemical alteration as underlying the duplication of the germ and the accompanying deformities of the resulting double embryos. The nature of the alteration of the water in which these embryos have developed is, of course, unknown and the factors responsible for their developmental anomalies are, undoubtedly, left altogether to speculation. It seems not improbable, however, that in such cases the osmotic pressure of the water may be increased by the presence in it of some (otherwise perhaps not injurious) metallic salts, or possibly blastotomy here results from increased temperature or "old age" of the eggs (cf. pp. 319-321 and 327).

that on examination of sections of some double monsters resulting from these experiments conditions are found which suggest that the duplication of the embryonic anlage was due to dissociation by the combined action of both chemical alteration and osmotic pressure (blastolysis).

The following cases may serve as illustrative examples of the apparent effects of such action.

In figure 11 is presented a 'parasitic' duplicity which in toto made the impression of a greatly deformed craniothoracopagus. Examination of sections practically confirms this diagnosis, except for the incompleteness in the duplication of the head.

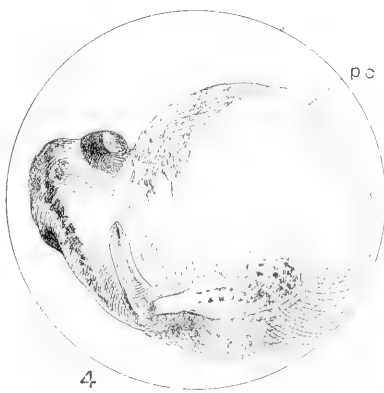
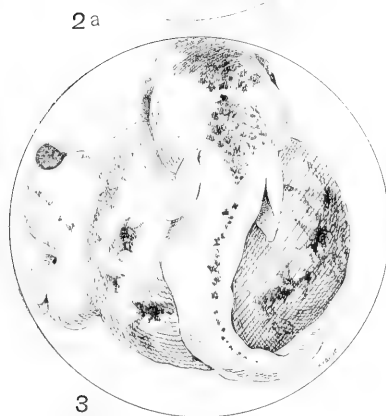
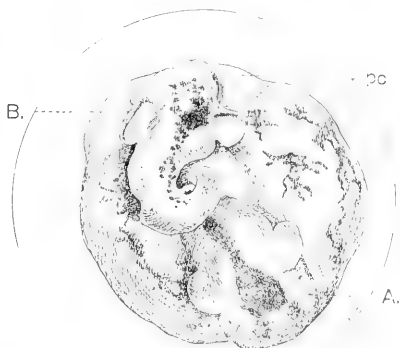
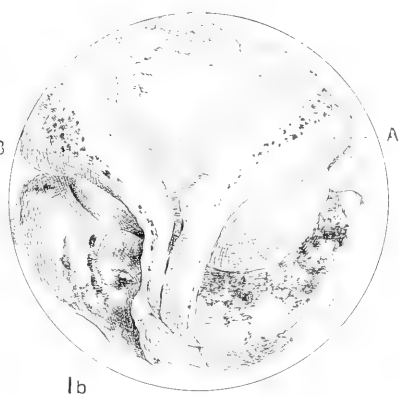
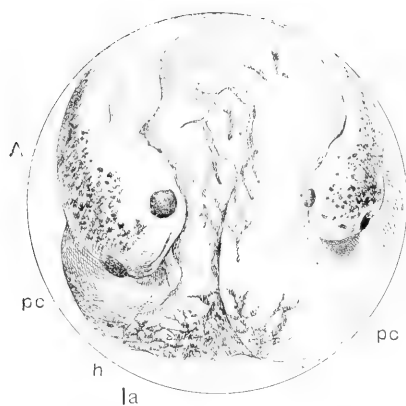
The transverse sections ( $6\ \mu$  in thickness) being somewhat oblique, those farthest anteriorly contain more of the right than of the left side of the duplicity. A large lens is noted in them, an olfactory pit and a distorted, unlobed fore-brain. The lens (but no eye on this side) was observed already in toto and was mistaken for a 'free lens.' In further sections, however, there comes into view in apposition to it a very small optic cup (fig. 14). A very small, lens-like body (*l*) can also be observed in several sections at this level between the ill-differentiated optic cup and the large lens. On following the sections posteriorly the eye of the left side gradually comes into view (figs. 14 and 15), while the optic cup of the right side becomes more and more elongate and is noted still to be in direct connection with the brain, no optic stalk having been formed. In sections still further posteriorly this eye is very plainly seen to be broken up (dissociated) into several parts—one of which at the base of the brain with which it is connected, has made a feeble attempt towards differentiation of the retinal layers,

Fig. 1 (a and b) Anterior and posterior views of slightly conjoined twins, *A* the larger, *B* the smaller one, From acetone solution (40 cc. gram-molecular to 50 cc. of sea-water), 19 days old, *h.*, heart, *pc.*, pericardial vesicle.

Fig. 2 (a and b) Two views of slightly conjoined double embryo, *A* the larger, *B* the smaller one. From butyric acid solution (10 cc. of a 1/16 gram-molecular solution added to 50 cc. of sea-water), 13 days old, *pc.*, pericardial vesicle.

Fig. 3 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old.

Fig. 4 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old.



rods and cones being discernible (fig. 15). At this level the brain has increased in size and presents a very distorted mass of nervous tissue in which cellular areas predominate. Still further posteriorly the buried optic cup of the right side is observed to gradually 'reconstitute' itself. It is very large at this level and its structure still betrays evidence of dissociation (*o.c.* fig. 16). The brain increases in size with every successive section (fig. 17). Some parts of it give the impression of retinal tissue, but no certainty can be felt regarding the latter point.

The duplication of the brain becomes distinct at a level where the (dissociated) part of the larger component is on one side intimately fused with the medulla of the smaller component ('parasite'—fig. 18). Two notochords and two (incomplete) alimentary tracts are also noted on the monster's right and left side respectively (figs. 17 and 18). Sections passing through the larger component's tail show distinctly (fig. 19) that the partial doubling of the central nervous system has come about through a splitting of one anlage by dissociation.

A clue to the genesis of the described conditions is found in anterior sections. Here the epithelium is dissociated (*d.e.*, fig. 14) in the region of the mouth and in a part of the blastoderm. The single oral cavity of the double monster is not continuous, but anteriorly partly occluded by the dissociated epithelium of the mouth (*d.m.*, fig. 15) and posteriorly by the dissociated posterior part of the optic cup, while posteriorly to the latter the lumen of the mouth appears as a narrow slit, being 'plugged' by dissociated epithelium. The latter as well as all of the epithelium of the mouth consists of large, vesicular cells.

Turning, now, to a consideration of the mode of formation of this monster, it would appear improbable that it has arisen from separated blastomeres and subsequent fusion during the formation of the embryonic bodies. The condition of the nervous system described above would seem to indicate clearly that at first one embryonic anlage has existed until probably about the time of formation of the embryonic body when the greater part has apparently been doubled by dissociation.

As an other case where the duplication has apparently occurred at a late stage (during the formation of the embryonic body) may be regarded the embryo (mesocatadidymus?) presented in figure 13.

This is a monster to which I attach much significance, because it most strikingly suggests the syngenesi of 'defects' with 'excess.' For its anterior part is highly deformed and defective while the greater part of the trunk and the tail—the former only partly—are duplicated, the spinal cord and notochord being multiple.

Examination of sections shows these conditions in a very convincing manner.

With the description of the defects of the head we shall not concern ourselves here, as it has a special bearing on the origin and differentiation of the lens and is therefore reserved for another paper. Suffice it to state, however, that the central nervous system is very defective throughout for about the anterior third of the body, i.e., to about the level of a plane passing transversely through the posterior part of the (only) pectoral fin. Just about from this level onwards posteriorly the spinal cord, in successive transverse sections, is observed at first to flatten and then to spread out more and more from side to side. It gradually increases in size and in many consecutive sections appears to be vacuolated and to exhibit evidence of cytolytic degeneration (fig. 20). The latter now becomes more and more conspicuous with each consecutive section (fig. 21), cellular elements disappearing more and more, until in some sections not much more than the framework of supporting tissue is left. The nuclear débris of the chromatolyzed cells can be observed at quite a distance from this degenerated part of the cord.

This has then, evidently, been the part which suffered the greatest injury from chemical alteration and subsequently presented a point of least resistance to the action of osmotic pressure. For from this level on the spinal cord divides at first into two and then into more parts. The notochord is also multiplied, while in sections at a still more posterior level the

body of the embryo is observed also externally to be partly duplicated (figs. 21 and 22). The musculature is not doubled; yet there is a decided increase of it in bulk. The alimentary tract is also single and even incomplete, some of its parts lacking altogether. In sections through about the posterior fourth of the embryo there appear just about the level where the intestine ends, two large cavities lined with endothelium whose genesis and morphological significance I am unable to interpret.

The genesis of the chief morphological deviations of this embryo might be imagined in the following manner:

The part of the embryo which was to form the head has sustained the greatest destructive alterations from the sojourn in the toxic solution. Hence the extreme malformation and the defects of this part of the body. The bilateral parts of the germ-ring have apparently—owing to osmotic pressure—failed at first to fuse completely, this fusion occurring only later when some anlagen have, in this way, been doubled. The increase in osmotic pressure has also most likely contributed to the fragmentation and dispersion of some anlagen, as is evidenced by the condition of the notochord and the spinal cord.<sup>16</sup>

While the embryo is not a double monster in the strict sense of the word, it well illustrates the manner in which—according to Fischel's suggestion—certain double embryos may arise during the formation of the embryonic body. It would also seem to offer strong support to our contention that the same

Fig. 5 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old.

Fig. 6 (a and b) From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 16 days old; *i.e.*, 'isolated eye.'

Fig. 7 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old.

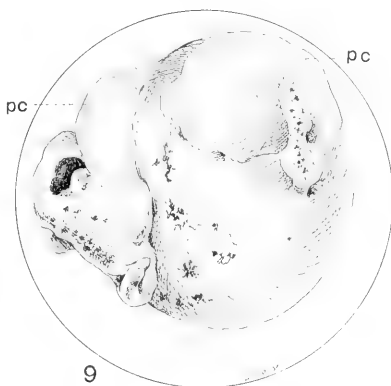
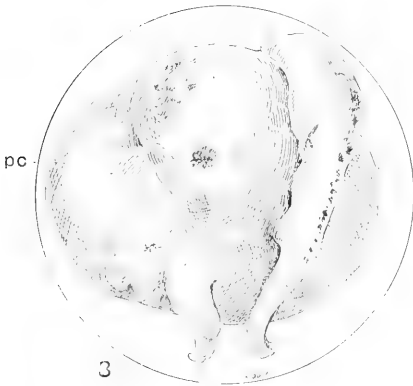
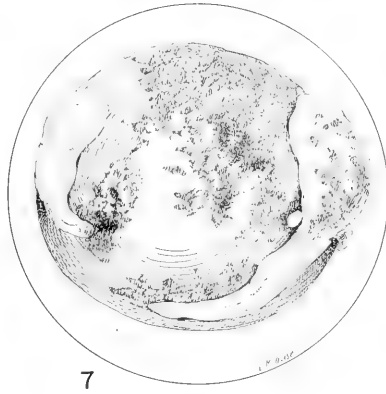
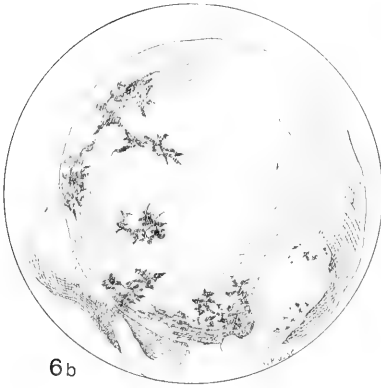
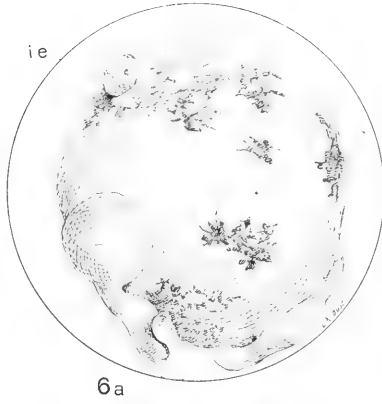
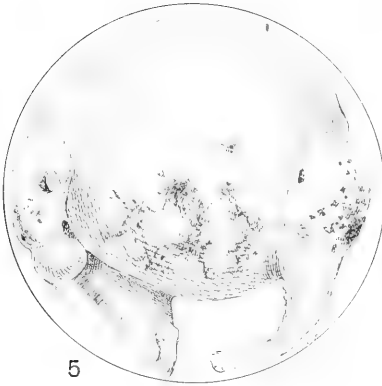
Fig. 8 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old, *pc.*, pericardial vesicle.

Fig. 9 From acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), one of the 'twins' amorphous, 14 days old, *pc.*, pericardial vesicle.

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<sup>16</sup> Neither verbal description nor the accompanying figures (of necessity limited in number) are adequate to give a complete picture of these conditions. To obtain an adequate idea of them and the dissociation that underlies them and is apparent in the sections, it is necessary to examine the latter in the series.





morphogenetic factor—blastolysis—is apparently responsible for both the ‘monstra per defectum’ and the double monsters and other ‘monstra per excessum.’

The next example which we have chosen for the demonstration of the apparent syngensis of both these deviations from the norm may now follow.

In figure 12 is presented an egg with a large ‘pericardial’ vesicle, dense vascularization of the yolk-sac and a very curiously misshapen embryonic mass which in toto was with some doubt interpreted as a dwarfed and highly malformed double monster. Microscopic examination of sections fully confirms this interpretation.

The first sections pass through the anterior part of the larger component only. They show (fig. 23) a dissociated tissue of an indifferent character in which are embedded two lenses, *l* and *l*<sub>1</sub>. Further sections show that this tissue mass increases in density posteriorly and that it is the anterior, greatly dissociated, part of the single optic cup which eventually comes fully into view surrounding on all sides the larger one of the two lenses (fig. 24). The brain of this component is unilobed, very irregular in shape and unusually small, while the eye is very large. The unusual shape of the latter (fig. 25) as well as also the fact that the ill-differentiated layer of rods and cones does not (as it should) appear throughout the entire outer margin of the optic cup wall, but is observed for a considerable stretch to merge into the other layers of the retina, suggests that the eye has been formed out of a dissociated anlage. The above mentioned dissociated optic tissue mass anterior to the eye adds considerable weight to this evidence of blastolytic action.

The smaller component appears first in the twelfth transverse section. It is also possessed of one eye only which is rather small anteriorly and very irregular in shape. In more posterior sections, however, this optic cup gradually increases to an unusually large size, (*o.c.*, fig. 26) it being almost as large as the brain of the component. The latter is unilobed, very irregular in shape and relatively very large.

On following the sections further the brain of the larger component is observed to increase to a very large size (figs. 26 and 27). Cellular areas predominate in it and are strangely intermingled with fibrous areas. It is difficult to escape the impression that distortions of this nature can have resulted from anything but a disorganization of parts of the primordium and subsequent processes of regulation.

At this level there can be observed in the larger component also a very small, vestigial ear vesicle (figs. 26 and 27). More posteriorly the notochord of this component is doubled, thus indicating dissociation of its anlage at an earlier stage of development. The (rudimentary) tail of this component comes into view in the last sections. A rudimentary tail is also observed in sections passing through the last part of the smaller component (fig. 27).

The internal organs of both components are almost entirely obliterated.

A point of interest is presented also by the epidermis of both components on the side on which they are conjoined. A cluster of large cells (*d.e.* figs. 25, 26 and 27) is noted here to come off directly from the epidermis. The large size of the cells would seem to indicate that having through dissociation lost their correlation with the epithelium of which they originally formed a part, there was no restriction to their expansive growth. In some sections the nuclei of these cells exhibit evidence of chromatolysis, thus suggesting that chemical alteration may have partly been responsible for dissociation (chemical blastolysis).

Turning, now, to the morphogenesis of this monster, we are justified, I believe, in assuming that it is a product of the combined action of, both osmotic and chemical, blastolysis. The former ('blastotomy') may by separating blastomeres (of the two-celled stage) have severed their mutual correlation, thus allowing each to develop independently. In the course of further development, however, the two, apparently not widely separated, embryonic primordia came into contact along their lateral surfaces where they coalesced.

The deformities and great defects (cyclopia) exhibited by both components are due to loss of embryonic substance by the double primordia owing largely to destruction by chemical alteration. The parts surviving this destruction have after regulation developed into two dwarfed, highly defective bodies.

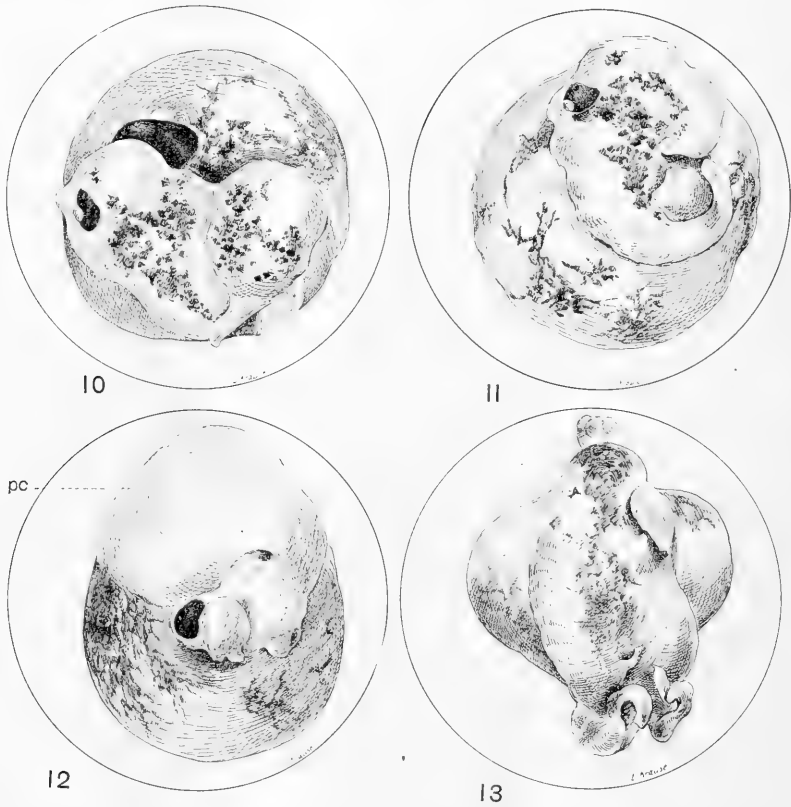


Fig. 10 'Parasitic' double monster from acetone solution (35 cc. of gram-molecular to 50 cc. of sea water), 14 days old. (Note the protruding, dissociated eyes of the 'autosite'.)

Fig. 11 Parasitic double monster from acetone solution (35 cc. gram-molecular to 50 cc. of sea-water), 14 days old.

Fig. 12 Dwarfed double monster from acetone solution (25 cc. gram-molecular to 50 cc. of sea-water), 15 days old, *pc.*, pericardial vesicle.

Fig. 13 Mesocatadidymus (?) from acetone solution (30 cc. gram-molecular to 50 cc. of sea-water), 26 days old.

A number of other duplicities have been recorded which also are *monstra et per defectum et per excessum* (figs. 1 to 10). Their genesis is, in all probability, due to the same factors that have been assumed for the monsters above described. Thus in the monster illustrated in figure 9, presenting double embryos of strikingly unequal size and greatly deformed, one of the 'twins' being amorphous, on microscopic examination indications are found of chemical alteration, while in the duplicity of figure 6 in which an isolated head fragment with a well differentiated eye (ascertained by microscopic examination) is observed at a considerable distance from either of the 'twins', action of osmotic pressure is apparent already on examination *in toto*, if conclusions from analogous observations in invertebrates (cf. for instance, Loeb, l.c.) be permitted.

The smaller one of the double embryos of figure 8 when examined in sections is observed to lack the anterior part of the head, the otic capsules appearing in the anterior-most sections. This is one of the most conspicuous defects of this component and, in my opinion, no error will be committed by assuming that the factor which brought about the duplication of the originally single embryonic primordium (osmotic pressure) may have contributed to the genesis of this and other defects by splitting off the chemically incapacitated parts. In the other double embryos recorded in our experiments which obviously are, at the same time also *monstra per defectum* microscopic examination of sections would, undoubtedly, furnish indications of the combined action of chemical alteration and osmotic pressure as underlying the doubling of primordia and the origin of the defects.

#### THEORETICAL REMARKS AND CONCLUSIONS

As pointed out repeatedly in the preceding pages no direct evidence is available for this combined action of osmotic pressure and chemical alteration. It may, therefore, seem desirable to inquire in how far their assumption is justified.

Nearly all duplicities of our experiments have resulted from the addition to sea-water (50 cc.) of a varied quantity (30 cc.

or 35 cc. or 40 cc.) of a gram-molecular solution (in distilled water) of acetone, only a very few resulting from the employment of butyric acid. Considering the molecular weights of sea-water on the one hand and of acetone on the other hand, it is at once evident that by the addition of the acetone solution to sea-water the latter was quite appreciably diluted. In other words, as far at least as this physical condition is concerned, our experiments with acetone are in principle similar to the experiments performed by Loeb (l.c.) on sea-urchin eggs by diluting the sea-water with fresh water. In both instances the osmotic pressure of the medium surrounding the eggs was lowered, although less so in my experiments than in those of Loeb. Since, in this way, the difference between the internal osmotic pressure (of the eggs) and the external osmotic pressure (of the surrounding medium) was modified in experiments which besides other monsters yielded a certain number of duplicities, there is no apparent reason to doubt that the origin of the latter is directly traceable to this modification.

It might perhaps be wondered why relatively few double embryos have resulted from the above described treatment of the eggs. This, however, can, at least partly, be accounted for. The egg of *Fundulus heteroclitus*, well known for its hardness,<sup>17</sup> is relatively little susceptible to physical insults, and it is possible that the dilution of sea-water by the addition to it of such quantities of acetone as I have employed, is not sufficient to cause osmotic blastolysis ('blastotomy'—Bataillon, l.c.) except in a very few eggs. Greater dilution of the sea-water by the addition of quantities of acetone greater than those employed in these experiments is, however, impracticable, for, in that case the degree of chemical action would be higher, and owing to it the mortality would, according to my experience, increase greatly and the few surviving eggs, having sustained so much chemical alteration, would result only in dwarfed, shapeless embryonic masses and fragments. The paucity of

<sup>17</sup> In a number of experiments performed in 1915 in which eggs of *Fundulus heteroclitus* were subjected to a strong centrifugal force, no alteration of development or any injury resulted.

double monsters in our experiments can, accordingly, not serve as an argument against the justification of our assumption of osmotic pressure as an important factor underlying their morphogenesis.

The second component factor of blastolysis, chemical alteration—is also largely inferred. However, this inference would seem to be well justified, if the action of acetone, partly solvent and partly as a precipitant of lecithine, is considered. Owing to the effect of this action some groups of cells may be entirely destroyed, while others may be only, more or less, chemically modified, and thus lose much of their inherent capacity for differentiation (inhibition) as well as of the power to resist the action of osmotic pressure.

Besides the two factors mentioned above attention may be called to another factor which may play a contributory part to the effect we term blastolysis insofar as the latter may be facilitated by it. This is the age of the egg, counting from the time of its maturation.

It has been found by several observers that the viability of the eggs and their ability to develop in a typical manner decreases gradually after their maturation.

Thus Hertwig ('92) noted that unfertilized frog eggs retained (for some time after the animal had been killed) in the uterus (on ice), showed with each consecutive day a greater tendency to abnormal development, which in many of them resulted in 'spina bifida.' Like results have been obtained by him also if female frogs were separated from males long enough for the eggs to become over-mature.

Conklin's ('97) observations on the eggs of *Crepidula* are very striking and may therefore be best presented in his own words (p. 30):

. . . . when the adult *Crepidulas* are brought to the laboratory, and kept in the best possible conditions, the percentage of these abnormalities increases, and when the egg capsules are removed from the mantle cavity of the mother and kept in dishes of sea-water, the monstrosities increase to such an extent that after a few days not a single normally developing egg or embryo can be found.

Bataillon (l.c.) has observed a very marked tendency to fragmentation and formation of double embryos in eggs of *Petromyzon* which had been retained in the females for several days after stripping. Since no modification of the environment whatsoever was employed in this case, Bataillon speaks of 'blastotomie spontanée' as the factor responsible for duplication.

Of the causes underlying this apparent deterioration of 'stale eggs' Goldfarb ('16, '17 a and b) has made a systematic study. He finds that it is apparently due to a progressive increase in their permeability and to the thus resulting rise in the rate of their oxidation, as postulated long ago by J. Loeb.

I am inclined to attribute considerable importance to this factor in our experiments in which, usually, eggs of several females were fertilized together before they were subjected to the action of the environmental modification. In other words, the material of our experiments was of a varying viability and varying susceptibility, the 'younger' eggs being less susceptible than the 'older' ones. This variability cannot well be obviated in any experiments of an explorative nature. For a single female of *Fundulus heteroclitus* will not yield eggs enough for a coherent series of experiments in which the same environmental modification is employed, but differing in each experiment in degree or in the stage at which the eggs were subjected to it. Besides, it is well known that not all eggs spawned by, or stripped from, a female at the same time are of the same 'maturation age.' It is a common experience to find mature and immature eggs in the same female during the spawning season, a fact which well indicates that the mature eggs have not all matured at the same time.

To this variation in degree of susceptibility of the eggs may be due the enormous range in variation in the end-products of development—from apparently perfectly normal embryos to monstrosities of a most bewildering kind. The duplicities which were recorded in our experiments are an incident in this almost endless 'series.' They result primarily from duplication of the embryonic primordium by blastolysis. And they as well as all other monstrosities are expressions of the variation in the



degree of this action, for they, too, range from apparently symmetrical, well formed double embryos through various degrees of deformation to highly defective and grossly malformed double monsters (figs. 1 to 13).

Among some other points which would also seem to call for explanation is the often recorded unequal size of the components of a duplicity where two whole slightly conjoined or entirely separate embryos have developed (figs. 1, 2, 3, 4, 6, 7, 8, 9, 10, 11).<sup>18</sup> In my opinion the only explanation possible for this phenomenon is that apparently even less than one-half of the entire embryonic primordium is still totipotent in teleosts just as Eataillon (l.c.) has found this to be the case in *Petromyzon*. In the case of our double embryos I would further suggest that after the splitting of the germ into two parts one of them has apparently sustained further blastolytic lesion which, however, while dwarfing it in size, has not diminished its totipotency.

In this connection it may be well to consider also the sometimes very striking differences in degree of malformation between the components of a duplicity (figs. 2, 4, 8, 9, 10 and 11). In accordance with our interpretation of the genesis of malformations (Werber, '16) it will have to be assumed that the more deformed component was subject to blastolytic action in a higher degree than the less deformed one. The above mentioned difference in size between components of the same duplicity is just another effect of this simple principle. I am, however, unable to account for these differences in degree of action on duplicates of the same germ.

Yet these differences (both in size and degree of malformation) between components of the same duplicity I regard as important insofar as they would seem to furnish a clue to the morphogenesis of the so-called 'parasitic double monsters'. For, granting an early and intimate secondary fusion of primordia duplicated by blastolysis, and that one of these double

<sup>18</sup> Similar observations on very young double embryos have been made also by Klausner ('90) in the chick and in the lizard *Lacerta viridis* and by Daresté ('91) in the chick, while one such double embryo of the chick is in my possession.

germs has sustained much greater injury than the other, the end-product of further development will be a more or less well formed (sometimes even much deformed) embryo (the 'auto-site') with a sort of 'parasitic appendage'— the 'parasite.'<sup>19</sup>

This conception of the manner of formation of the 'parasitic' duplicities differs essentially from Wilder's ('08, pp. 363-364, ff.), for no resort is made to the improbable secondary degeneration of one (whole) of the already formed components of the dipopagus owing to—what briefly may be called—physiological dominance of the other component.<sup>20</sup>

\* \* \*

It may now be questioned in how far our conclusions regarding the origin of the duplicities in our experiments can be extended to those occurring in nature in other vertebrates and particularly in mammals. While I am, of course, disinclined to do so unconditionally, I think that with some restrictions this extension may be regarded as permissible. For the oft-assumed possibility, if not probability, that in nature, too, the primary causal agent of atypical development is a chemical one (probably due to some disturbances of metabolism during which substances are produced by the body otherwise foreign to it) can not be denied. The human organism—and possibly that may apply to other mammals and to Sauropsidae as well—is subject to disturbances of metabolism of both a serious and a relatively harmless nature. To the former may be counted nephritis, diabetes, jaundice and other diseases of metabolism, while as a relatively harmless, temporary disturbance of metabolism may be viewed the presence in the organism of certain substances of fatigue after muscular exertion (for instance, lactic acid or monopotassium phosphate) and perhaps some other products of transitory abnormal conditions. While the substances of the former owing to their continued more or less severe

<sup>19</sup> Fischel (l.c., p. 290) also suggests that these 'parasitische Anhänge' develop from primarily rudimentary anlagen.

<sup>20</sup> The differences between the 'parasitic appendages' and the foetal inclusions ('teratomata', 'embryomata', etc.) I regard, with Fischel, (l.c., pp. 290-299 ff) as secondary only. A discussion of this subject is deferred to a future publication.

action<sup>21</sup> on the ovum of a female suffering from any of these diseases may cause either its death and subsequent expulsion or any conceivable monstrosity, the latter, if altering the tonic-ity only of the blood, may by (osmotic) blastotomy produce slight defects or duplication of the embryonic primordium and thus in the latter case be responsible for the development of monozygotic twins, or any monozygotic, more or less well formed, 'symmetrical' duplicity.

It might further be questioned whether the analysis of the morphogenesis of our experimental fish duplicities as attempted in the preceeding pages on the basis of Fischel's theory is applicable to the higher vertebrates and mammals.

Sobotta ('14) has already pointed out that while it is very doubtful whether the first two blastomeres are equipotent and totipotent in mammals it may be regarded as hazardous to extend to the latter the conclusions reached regarding the morphogenesis of duplicities in lower animals. However, occasioned primarily by the recent researches of Fernandez ('09) and Newman and Patterson ('10) and Patterson ('13) he suggests hypothetically a stage (the 'embryonic blastomere' of the four-celled stage) whose parts after the next cleavage might be totipotent and thus capable of giving rise to monochorionic double embryos if separated.<sup>22</sup> Undoubtedly, in view of the meager data available for the earliest development in mammals, generalizations must be made with extreme caution. Yet, whatever the mammalian embryo-forming equivalent of the ovum of amphibia (or other animals in which the first blastomeres are totipotent) may be, there is, as Fischel (l.c.) has already pointed out, no apparent reason to expect that its prospective potency should be lower than that of the latter.

<sup>21</sup> Depending partly upon the 'maturation age' of the ovum at the time of its fertilization.

<sup>22</sup> For *Tatusia novemcincta*, on which Patterson's ('13) investigations were carried out, Sobotta assumes the separation of the four cells resulting from the second cleavage of the hypothetical 'embryonic blastomere.' Pending further investigations on the earliest stages of development in armadillo I should regard as questionable the analogy between the polyembryony in the latter and the occasional instances of 'twinning' in other mammals.

Granting, however, the possibility that up to the time of gastrulation the mammalian (and Sauropsid) ovum is capable of being divided into two or possibly more equi- and totipotent parts, it would remain to be shown that the parts thus separated may, as in *Petromyzon* and teleosts, be able to form any kind of duplicity, depending upon the degree of separation, the distance between the parts separated and the angle of their respective convergence. There is reason to believe that no differences should exist in this regard between the teleost ovum on the one hand and the Sauropsid and mammalian ovum on the other hand. For, as Fischel sets forth (l.c., p. 274), the processes leading to the formation of the embryonic body (separate anlagen for the head and trunk, the latter formed by concrescence of two lateral parts) being in principle very much the same in all vertebrates the morphogenesis of double monsters in teleosts may—at least with certain restrictions of secondary significance—be extended to amniotes and even to man.

These conclusions are, obviously, tentative and—in default of experimental tests in mammals—they will probably remain so for an indefinitely long time. Their advantage, however, besides offering a plausible explanation, is that they may help to keep alive further inquiries into the, so far inapproachable, problem of diplogenesis ('twinning') in amniotes.

It is for this reason mainly of methodological advantage that, —in default of evidence to the contrary—I am inclined to hold to the views above expressed in preference to Wilder's speculations<sup>23</sup> on the subject. For, while I can find no fault with this writer's reasoning, I consider that his separation of the double monsters (*sensu stricto*) from well formed, symmetrical duplicities ('orderly beings—cosmobia') and the assumed mysterious origin of the latter by a sort of mutation due to 'germinal variation' are both unnecessary and arbitrary. The lack of deformities in 'Cosmobia' as well as in viable monozygotic twins would rather seem to indicate that while they may be developmental products of primordia duplicated by blastotomy, they

<sup>23</sup> A view similar to that of Wilder's seems to have been advanced also by Bolk ('06), whose paper is, unfortunately, inaccessible to me.

have apparently sustained no destruction due to this or any other factor. The symmetry in anatomical details observed in 'Cosmobia' may, I venture to say, be only an incident—symmetrical blastotomy and the subsequent undisturbed development of the primordia which have, thus incidentally, remained equipotent. Conversely we may assume that where (osmotic) blastotomy or blastolysis (both osmotic and chemical) causes destruction of some areas of the germ, any monster and—if the germ has also been split into two more or less equivalent parts—any deformed duplicity may result from further development. The distinction between these two kinds of duplicities is, accordingly, mainly one of degree; it is a quantitative difference rather than a qualitative one and it may depend upon the specific action (either chemical or physical or both chemical and physical) of the (probably chemical) agent primarily responsible for the deviation from the typical course of development.

Moreover, this quantitative difference may, as pointed out above, depend also upon the 'age' of the egg, the 'younger' eggs being hardier and more capable of surviving blastotomy without sustaining fatal lesions, while the 'older' ones with a tendency to cytolytic fragmentation may apparently be (in a varying degree) more subject to such lesions produced by blastotomy or blastolysis.

The 'old age' of the egg is, undoubtedly, a germinal variation, but this 'variation' is in the nature of a pathological change and the causes underlying the latter may well be within the bounds of discovery, as is suggested by the recent researches of Goldfarb (l.c.). The germinal variation, however, assumed by Wilder is not a pathological condition and being nothing else that could intelligibly be defined, it automatically puts a stop to further inquiry into the primary causes underlying diplogenesis in nature.

While I am not at all inclined to underestimate the almost insuperable difficulties presented by the problem, I cannot on the other hand subscribe to Wilder's skepticism voiced in the following characteristic remark ('08, p. 428):

. . . . even though the monsters resulting from the present or future experiments, in which the cause is applied after the formation of the germ and is therefore secondary, be found upon careful examination to be definite Cosmobia, it will prove only that such monsters *can* be thus produced and not that Nature *does* produce them in the same way.

It is, obviously, difficult to deny the justifiability of this argument, but on the other hand—I think Professor Wilder will admit—it practically amounts to questioning the value of many explorative experiments in biology.

May 30, 1917.

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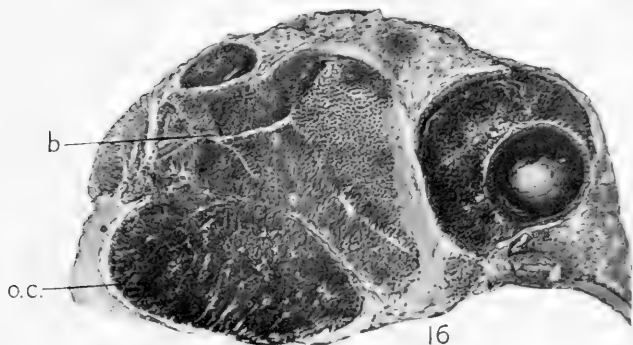
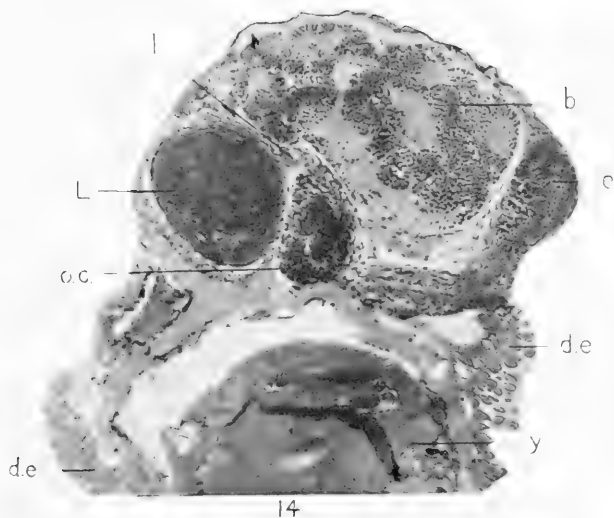
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## PLATE 1

## EXPLANATION OF FIGURES

14, 15 and 16 Transverse sections through 'parasitic double monster' of figure 11. *L.*, lens; *l.*, small lentoid body; *b.*, brain; *e.*, anterior-most part of eye; *d.e.*, dissociated epidermis; *o.c.*, optic cup; *d.o.*, dissociated optic cup; *d.m.*, dissociated epithelium of mouth; *y.*, yolk.  $\times 105$ .

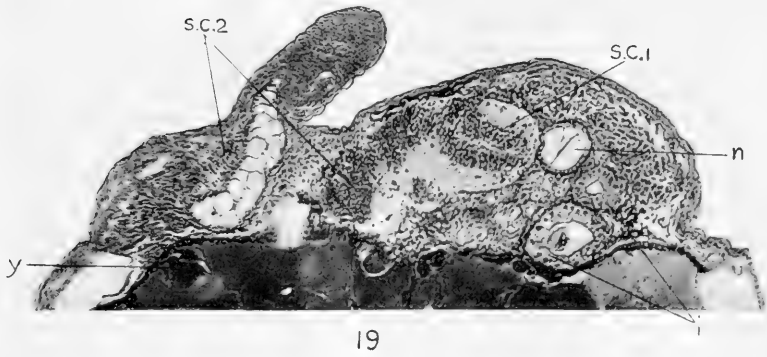
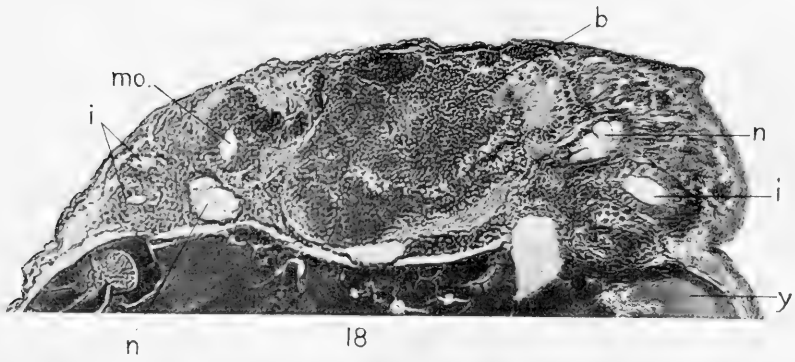
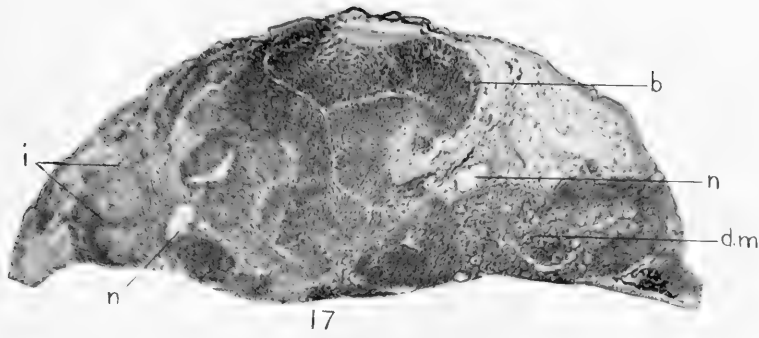




## PLATE 2

### EXPLANATION OF FIGURES

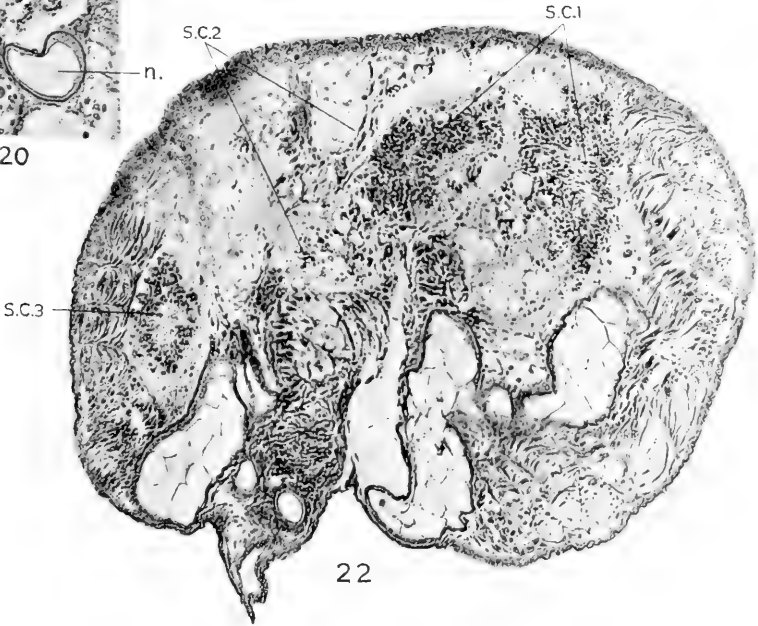
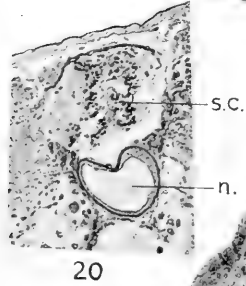
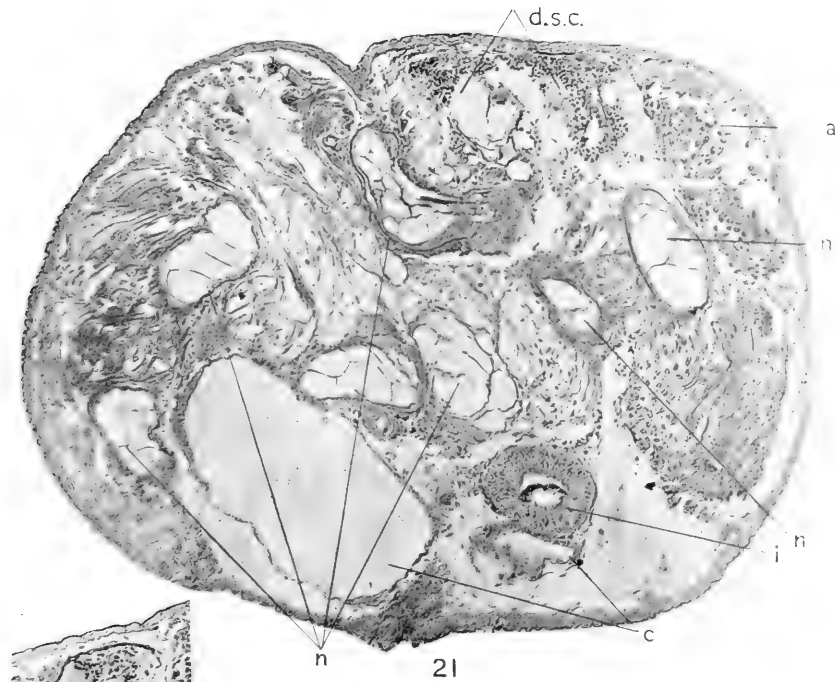
17, 18 and 19 More posterior sections through the embryo of figure 11. *b.*, brain; *n.*, notochord; *i.*, intestine; *d.m.*, dissociated epithelium of mouth; *m.o.*, medulla oblongata of 'parasite'; *s.c.* spinal cord of 'autosite'; *s.c.*<sub>1</sub>, spinal cord of 'parasite'; *y.*, yolk.  $\times 105$ .



## PLATE 3

### EXPLANATION OF FIGURES

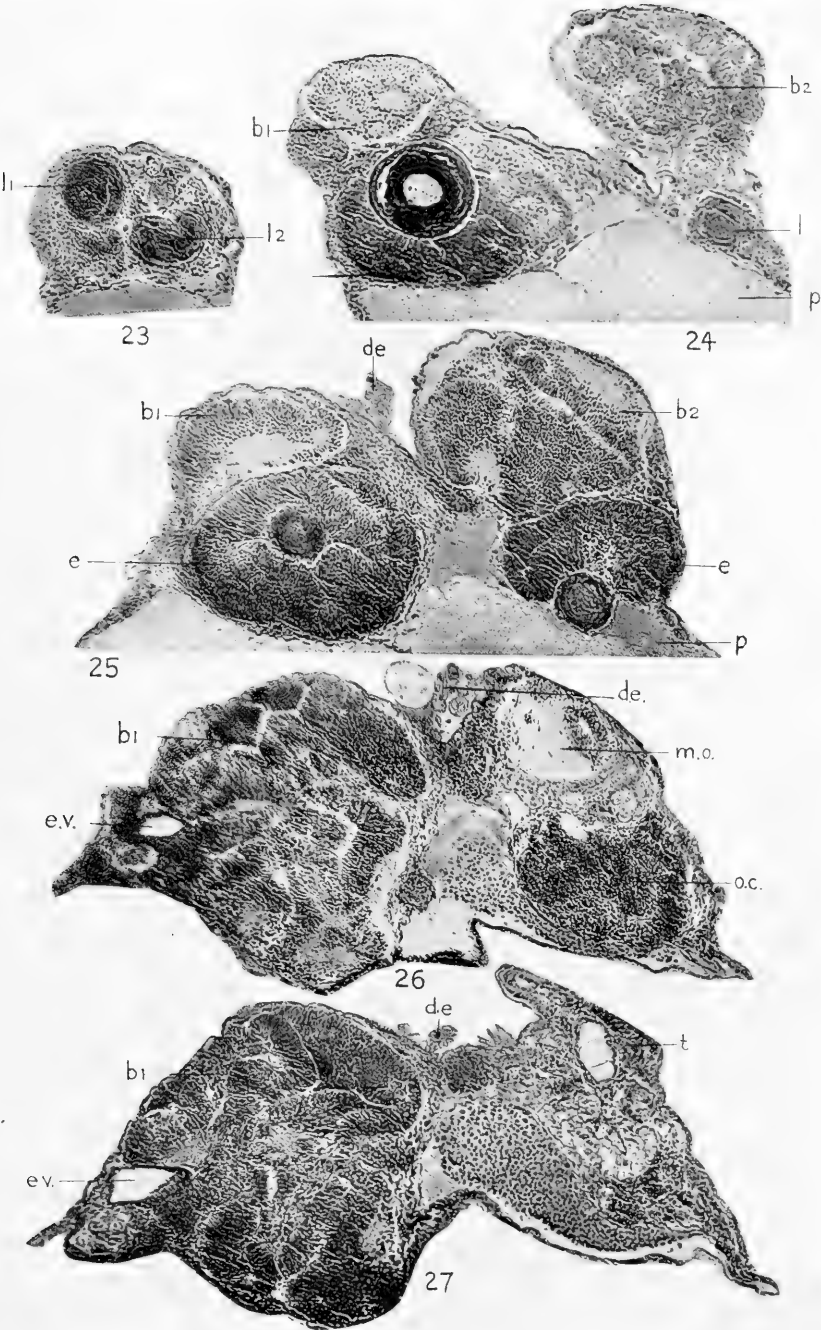
20, 21 and 22 Transverse sections through embryo of figure 13, *n.*, notochord; *s.c.*, spinal cord; *d.s.c.*, dissociated and partly 'degenerated' spinal cord; *s.c.*<sub>1</sub>, *s.c.*<sub>2</sub> and *s.c.*<sub>3</sub>, dissociated, multiple spinal cord, cavities lined with endothelium.  
× 105.



## PLATE 4

### EXPLANATION OF FIGURES

23, 24, 25, 26 and 27 Transverse sections through dwarfed double embryo of figure 12. *b*<sub>1</sub>, brain of the larger component; *b*<sub>2</sub>, brain of the smaller component; *l*<sub>1</sub>, and *l*<sub>2</sub> lenses in anterior-most, greatly dissociated part of the eye of the larger component; *l*, lens (smaller component); *e*, eye; *d.e.*, dissociated epidermis; *m.o.*, medulla oblongata of smaller component; *e.v.*, rudimentary ear vesicle; *o.c.*, posterior-most, greatly dissociated part of the optic cup of the smaller component; *p.*, plasma in pericardial vesicle; *t.*, tail of smaller component. × 105.







## CONTRIBUTIONS TO THE STUDY OF CELL MECHANICS

### II. MONASTER EGGS AND NARCOTIZED EGGS<sup>1</sup>

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### ERRATA

THE JOURNAL OF EXPERIMENTAL ZOOLOGY, volume 24, number 2, November, 1917. In the last line of footnote 15 on page 415, the cross reference to pp. 319-321 and 327 should be (Cf. 427-428 and 433).

Paste this slip facing page 415 when the volume is bound.

experiments made in an attempt to understand the meaning of the behavior of certain cell elements. In making an analysis of the observed facts, we have been brought face to face with so many mooted questions of cell mechanics, which have been left unsolved, that it is best, perhaps, to define the view-point from which the present research was undertaken. Cytologists

<sup>1</sup> The present work was carried on at the Zoological Station at Naples, at the Woods Hole Marine Biological Laboratory, and at the Osborn Zoological Laboratory. Contribution No. 136 from the Zoological Laboratory, University of Texas.



CONTRIBUTIONS TO THE STUDY OF CELL  
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TEN TEXT FIGURES AND FIVE PLATES

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INTRODUCTION

The present contribution embodies the results of a microscopic study of monaster eggs in sea urchins, together with some experiments made in an attempt to understand the meaning of the behavior of certain cell elements. In making an analysis of the observed facts, we have been brought face to face with so many mooted questions of cell mechanics, which have been left unsolved, that it is best, perhaps, to define the view-point from which the present research was undertaken. Cytologists

<sup>1</sup> The present work was carried on at the Zoological Station at Naples, at the Woods Hole Marine Biological Laboratory, and at the Osborn Zoological Laboratory. Contribution No. 136 from the Zoological Laboratory, University of Texas.

seem to be generally agreed that, in the ultimate analysis, the problem of cell division is one for the colloidal chemist. On the other hand, the morphological phenomena, which are expressions of chemical and physical changes, are extremely varied in cells of different types, and any one familiar with the vast amount of cytological literature which has accumulated the last twenty years, must realize that scarcely any two authors are in agreement as to the really essential features with which the chemist must work. Simple morphological studies, while extending our conception of the cell in its varied activities, cannot bring us nearer to this goal. It is only by experimental studies, such as the Hertwigs, Boveri, Wilson, Conklin, Morgan, and others have given us that real progress has been made. These authors have shown that much light may be gained by subjecting one type of cell, such as an egg cell, to abnormal conditions. When this is done the forces, which in normal cleavage work synchronously, run their courses independently and the experimental cytologist may sift out the essential from the non-essential, and eventually formulate the questions which the physical chemist must solve, as soon as the nature and physical behavior of colloids are more thoroughly understood.

The present study was undertaken with the hope that an analysis of monaster eggs would enable me to clear up some of the confusion which has existed in cytology. The first part of this work was undertaken at the suggestion of Theodor Boveri, at Naples, during the spring of 1914. Here the observation on the living eggs were made and material for cytological study preserved. The experiments with the narcotic were made at Woods Hole, Mass. While in Naples I enjoyed the privilege of Professor Boveri's experience and advice. The observations on the living eggs were known to him but the results of the cytological study together with the conclusions I have drawn from them, were not completed until after his death.

## MONASTER EGGS

Although monaster eggs were known long before, Boveri seems to have been the first to realize the value of such eggs in analytical studies of cell dynamics and both he and his wife published papers dealing with the external behavior of the eggs (Th. Boveri, '03), and the behavior of chromosomes, centrosomes and other cell parts (M. Boveri, '03). Neither of these authors, however, had undertaken a study of the movements of the protoplasm which are such a conspicuous feature of monaster eggs; especial attention was therefore given to this in the present work.

A great variety of agents act upon the normal sea urchin eggs in such a way that only one division center appears when the division cycle is initiated. Numerous chemicals produce monasters, strychnine (Hertwigs, '87),  $MgCl_2$  (Wilson, '01 a), carbon dioxide (Herbst, '14). Many narcotics do the same. Chloral hydrate (Hertwigs, '87), ether (Wilson, '01 b), phenyl urethane (Painter, '15). Mechanical stimuli act often in the same way. Thus Boveri found that by shaking eggs a few minutes after fertilization, a good percentage of monaster eggs often resulted.

The last named method was employed, as it was the simplest, and introduced no additional factor, such as the use of chemicals might have done. As soon as the fertilization membrane appeared, in eggs artificially fertilized, they were placed in a test tube, with as little water as possible and shaken violently by hand for twenty seconds. Precaution was taken to keep the warmth of the hands from increasing the temperature of the eggs, and after shaking they were placed in shallow dishes and allowed to develop.

The production of monasters is capricious and seems to depend as much upon the condition of the eggs themselves as on any other factor. If one treats two sets of eggs from different females in just the same way, shaking the same amount and at the same time, in one set one may get ten per cent monasters, and in the other only a few or none at all. Experiments

were made to determine what was the best time to shake the eggs. The results of these different experiments are given in the tables below. From these it will be seen that when eggs were shaken between four and eight minutes after fertilization, the largest number of monasters were produced.

The extreme variability which monaster eggs show in their later behavior has been noted by Wilson ('01 a), Boveri ('03) and myself ('15). For the sake of clearness I have given the main paths of divergence, as I have found them, in the following scheme.

TABLE 1  
*Experiment of March 2*

MINUTES AFTER FERTILIZATION SHAKEN	NUMBER OF NORMAL	NUMBER OF MONASTER	PERCENTAGE OF MONASTER
2	391	9	2.2
4	411	83	18.0
6	385	115	23.0
8	387	125	24.0
10	470	44	9.0
12	410	5	1.0

TABLE 2  
*Experiment of March 4*

MINUTES AFTER FERTILIZATION SHAKEN	NUMBER OF NORMAL	NUMBER OF MONASTER	PERCENTAGE OF MONASTER
4	221	48	21.0
6	480	26	5.2
8	200	5	2.5
10	500	25	5.0

TABLE 3  
*Experiment of March 11*

MINUTES AFTER FERTILIZATION SHAKEN	NUMBER OF NORMAL	NUMBER OF MONASTER	PERCENTAGE OF MONASTER
3	380	12	3.0
5	395	51	10.0
7	296	32	10.0
9	202	27	10.0
11	300	0	0

Monaster eggs may:

1. Divide early without passing through a period of protoplasmic movement. Frequently spiral asters are found in such eggs.
2. Pass through division cycle and:—
  - (a) form amphiasters, divide and develop normally.
  - (b) form tetrasters or triasters.
  - (c) form multiple asters.
  - (d) form monasters.

In the following account the description of the living egg will first be given and then the results of the study of sectioned material.

*Changes in living egg*

The monocentric condition is a normal phase in the development of an amphiaster, (Boveri '00, p. 155). Consequently, monaster eggs cannot be certainly distinguished until the majority of the controls are in some late phase of the first division cycle. By this time the single aster will have reached considerable size, and is seen approximately in the middle of the egg with radiations running in all directions. The size of the aster is variable.

As the control eggs divide, the monaster eggs show little change except a slow increase in the size of the clear centrosphere. As a few of the controls are entering the early phases of the second cleavage, some of the monaster eggs undergo changes which lead to the formation of an amphiaster, and such eggs divide. The vast majority of eggs, however, show no change until about half the controls are in the 4-cell stage. The single aster, which by this time is very large, begins to undergo a striking change. It slowly becomes flattened on one side, then concave, and finally saucer-shaped. As this is going on it moves through the egg's substance until it lies well against one side of the egg (fig. 1). The convex side of the aster goes forward, during the movement and the fibers on the concave side seem to reach nearly to the opposite side of the egg. There is a great amount of variability in the amount of the flattening, and of the retreat of the aster, as a glance at text figures B, C and D will show.

As the aster assumes its new position, with high power, one sees little clear vesicles lying on the edge of the centrosphere on the concave side. These are the chromosome vesicles which slowly fuse to form one nucleus, as the movement of the protoplasm, described below, sets in.

Following directly on these changes in the aster and in the chromosomes, we have a series of phenomena which involves the protoplasm of the egg. This is first seen in the ectoplasm (or 'Verbindungsmembran' of Herbst, '00), which, on the side of the egg opposite the concave side of the aster, (fig. 1) begins to swell or blister. While this layer remains thin and difficult to see, except in dim light, on other parts of the egg, in the region opposite the aster it increases in thickness and frequently becomes granular and more or less opaque.

After the membrane swells, the whole area of protoplasm, which it covers, begins to undergo a series of changes of contour during which little ridges and depressions appear. These rapidly change into pseudopod-like processes (fig. 2. In this drawing as in the rest of the figures on this plate, the ectoplasmic layer is not shown) and the whole surface of the egg becomes involved in a series of slow protoplasmic movements. The mobility of the protoplasm is extraordinary. Figures 2, 3, 4, 5, 11, 12, 14 show typical cases in various phases of the movement. The egg becomes flattened as the movement progresses and the finger-like processes undergo rapid changes in shape like the pseudopods of an amoeba, and these processes may even be cut off entirely from the egg (figs. 4 and 5). In this event, however, they continue to undergo changes in shape for a short time.

As the radiations of the aster diminish in intensity and disappear, the protoplasmic movements begin to subside. The finger-like processes are withdrawn; the little protoplasmic balls again fuse with the egg, (figs. 12 and 14) and the whole egg becomes rounded out, in most cases, before the next division cycle is well advanced. The large excentric nucleus and the swollen ectoplasmic layer are the only features which show that the egg has passed through the monaster cycle.



The protoplasmic streaming is extremely variable not only in the eggs of different females, but in the eggs of one individual. The typical course of events is shown in the camera and free-hand sketches reproduced in figures 1 to 5 and 11, 12, 14. But in some eggs the aster does not flatten so much as to become concave, nor does it move very far in the egg substance. Going hand in hand with this, the movement of the protoplasm is not so severe. A full discussion of such eggs will be given later.

One very striking phenomenon which accompanies the protoplasmic movement, is the flow of the material from the cortical layers of the egg into the protoplasmic processes. This is best seen in heavily pigmented eggs, such as those of *Arbacia*, from which figure 11 was made. The same flow is seen in *Strongylocentrotus*, although it is not conspicuous because of the lack of pigment.

The time when the movement of the protoplasm is seen is extremely variable when measured in terms of the control eggs. It may be observed anywhere between the 2- and 8-cell stage. But in any single egg it only follows after certain definite changes in the aster and in the chromosomes. The length of time which the movement lasts is also variable. Sometimes the egg will be rounded up twenty minutes after the swelling of the membrane began, or it may be double that time before the period of movement is over.

The second division sets in, usually, just as the normal rounded form is being resumed (fig. 14) and the divergence which various eggs show in their behavior (see p. 449) begins.

In the large majority of cases, the second division cycle is accompanied by the formation of an amphiaster (figs. 12 and 14). Except for the excentric position of the spindle and the large asters, such eggs appear normal. When cytoplasmic cleavage sets in, however, we do not have the clean cutting through of the protoplasm. Instead, there is first a very pronounced swelling of the ectoplasmic layer in the cleavage plane, which amounts to a disintegration, often, on the side farthest away from the spindle (fig. A). This is followed by severe protoplasmic movements in the cleavage plane (figs. A<sub>1</sub>, A<sub>2</sub>

and figs. 7, 8) which eventually result in a separation of the two blastomeres, and frequently also, in the formation of a third (enucleated) ball, which may or may not fuse again with one of the blastomeres. We witness here again, during this division cycle, the formation of pseudopod-like processes (fig. 7) especially on the side farthest away from the spindle. Also, the two sister blastomeres become fully separated (fig. 8) when cleavage finally takes place.

Eventually the two blastomeres round up and the third division cycle sets in. The further history of these eggs has been described elsewhere (Painter, '15). Isolated eggs have given me some normal plutei. Boveri ('05) obtained the same results.

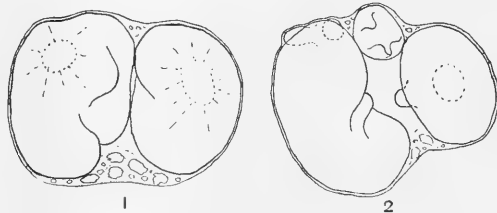


Fig. A

In a few monaster eggs, triasters and tetrasters are obtained at the second division cycle. As far as they were followed, they did not differ in their behavior, from dispermic eggs, and eggs isolated did not develop normally.

At the second division cycle the last two types of eggs mentioned on page 449, under c and d, can not be distinguished. One finds the reappearance of a single division center; this, however, is marked by the small size of the centrosphere, which rarely approaches, in my experience, the size of the monaster in the first division cycle. After a variable length of time, movements of the protoplasm set in which are essentially like those seen in the first division cycle of the monaster eggs except that the activity is not confined to one side of the egg but involves the whole or nearly the whole surface (fig. 6). Very frequently a rough division of the egg separates two blastomeres, (figs. F<sub>5</sub>, F<sub>6</sub>, or F<sub>7</sub>) but this is accompanied by a great deal of

protoplasmic streaming and the division is never permanent. Following the second division cycle, a third sets in. The movement of the second period is usually not completed by this time. The movements of the third period are even more severe, as a rule, than any of the earlier ones, and an endless number of bizarre shapes are seen. One very characteristic type seen is shown in figure 10. On such an egg little protoplasm drop-lets will be thrown off which do not fuse again with the mother egg, probably, because of the ectoplasmic layer has disintegrated by this time. Several more division cycles may follow (Wilson, '01 a, saw as many as 6 in one egg) but ultimately disintegration results (fig. 9).

The behavior both of the aster and of the protoplasm, as has been described in the foregoing pages, may be taken as typical of what we would find, in any given set of monaster eggs. But extreme variations are often found from the courses described above. The figures together with the descriptions in the following section will give some idea of the extent of these variations and will show that they are variations in degree and not in kind.

In figures B<sub>1</sub> to B<sub>6</sub>, the history of an egg is given in which the aster did not move to any appreciable degree. The egg was isolated when the controls were in the two-cell stage and it was a typical monaster (fig. B<sub>1</sub> drawn at 11.20). The egg underwent little change for the first ten minutes or so, then little chromosome vesicles began to appear and this was followed by a slight swelling of the ectoplasmic layer (fig. B<sub>2</sub>, 11.42). This was accompanied by a slight change in the contour of the egg's surface. At 11.50, the rays of the aster had disappeared but the protoplasm of the egg was still somewhat irregular in outline (fig. B<sub>3</sub>). At 12.08, an amphiaster was found in the egg. This led to a division during which the binding membrane was greatly swollen in the division plane and seemed to undergo a sort of disintegration, as may be seen by figure B<sub>5</sub>. The separation of the blastomeres was complete and was accompanied by the giving way of the cell wall, as was described for the eggs shown in figures 7 and 8. At 2.12, this egg had divided several

times (fig. B<sub>6</sub>), and the micromere which so often appears at this time is present (Painter '15).

This egg may be taken as typical in behavior in cases where the aster does not undergo a marked retreat. The swelling of the ectoplasmic layer and the movement of the protoplasm are both seen but neither are severe.

In figures C<sub>1</sub> to C<sub>6</sub> the history of an egg is shown, where the aster retreated only a short distance from the center. In figure

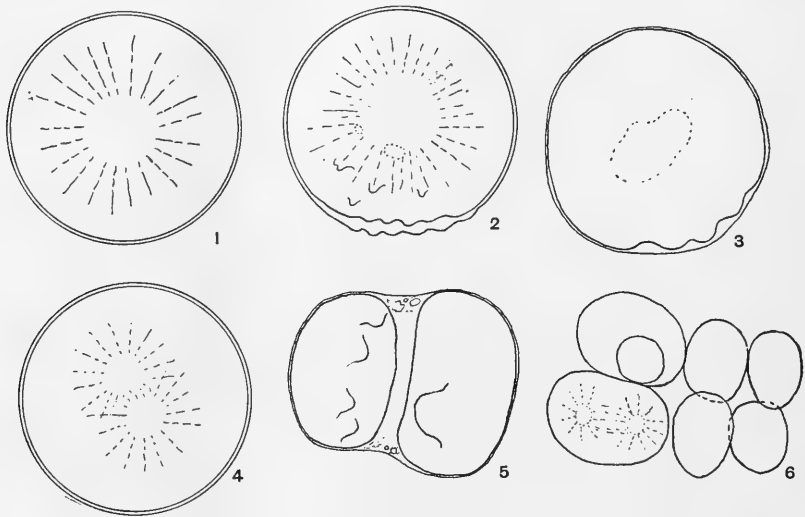


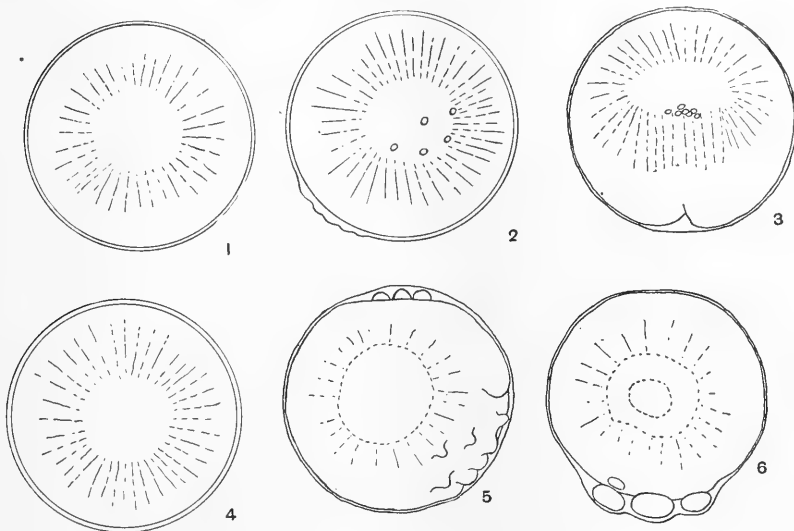
Fig. B

C<sub>1</sub> the typical monaster egg is seen, as it was isolated at 11.17. By 11.25 the chromosome vesicles were visible, and at 11.50 the aster occupied a position somewhat excentric to the center of the egg. When the vesicles were forming a swelling of the ectoplasmic layer was observed (fig. C<sub>2</sub>) and in figure C<sub>3</sub> we see the slight movement of the protoplasm. At 12.08 the monaster reappeared (C<sub>4</sub>) and at 12.25 the movement of the protoplasm can be seen (fig. C<sub>5</sub>). At 2.10 the egg had the condition shown in figure C<sub>6</sub>.

Figures D<sub>1</sub> to D<sub>4</sub> give the history of an egg in which the aster retreated farther than in the two cases just described. The swelling of the membrane together with the accompanying

protoplasmic movement is clearly shown in figure D<sub>2</sub>. An amphiaster appeared in this egg at the next division cycle (fig. D<sub>4</sub>).

These three cases, taken from notes in which the history of dozens of similar eggs is recorded, well illustrates the great



C  
Fig. C

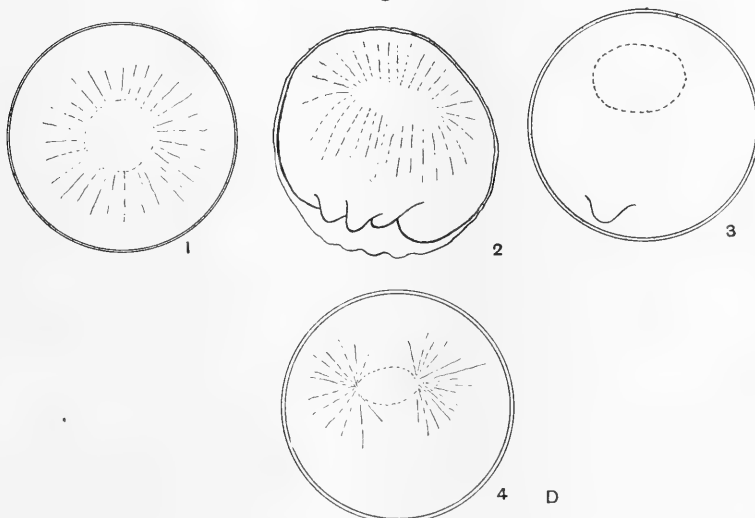


Fig. D

variability which the monaster eggs show in their behavior. These changes bear no direct time relation with the controls, the movements appear early or late, and continue for a shorter or longer time. (Direct evidence of this will be shown in the sections). In spite of this variation, however, there is a similarity in the behavior of all monaster eggs.

First there is always a swelling of the ectoplasmic layer before any sign of protoplasmic movement appears, and this swelling does not begin until the chromosome vesicles are being formed.

Second, the swelling invariably precedes the movement of the protoplasm.

Third, the severity of the protoplasmic movement seems to depend on the distance which the aster retreats from the center of the egg. When this retreat is extreme, we have a severe movement, figures 1 to 5, when it is not marked, there is little movement, figures B<sub>1</sub> to B<sub>6</sub> and D<sub>1</sub> to D<sub>6</sub>.

An additional feature of the behavior of the monaster eggs should be emphasized. This relates to the ectoplasmic layer. It not infrequently happens that the swelling of the membrane is accompanied by the appearance of little granular irregularly shaped bodies in the otherwise homogeneous layer. This almost invariably happens in the two cell stage, as is shown in figures B<sub>5</sub> and in A<sub>1</sub> and A<sub>2</sub>. When the two cells move apart, after the first division, this layer does not sink in as it does in the normal eggs. But it remains in the condition shown in the last figures named. Very frequently this swelling leads to the disintegration of the layer so that in the later stages, it is not present. As a result the blastomeres may become more or less scattered, as shown in figure B<sub>6</sub>, but this does not seem to interfere with the formation of blastulae or gastrulae.

#### *Internal changes*

In taking up the study of sectioned material of monaster eggs, two main points were to be determined, first, the cause for the divergence in the later behavior of the eggs, and, second, the exact condition of the aster and chromosomes just prior to

and during the periods of protoplasmic streaming. The detailed history of the aster and of the chromosome during the first monaster cycle had been worked out with such care by Mrs. Boveri that little was left to be added to this side of the problem.

Series of eggs, in which the per cent of monasters was high, were preserved at intervals of ten minutes or less, depending on the stage. The preservatives used were Boveri's picro-acetic mixture and sublimate acetic. After the usual treatment eggs were sectioned at 7 microns and stained in iron haematoxylin followed by light green. In practically all series whole mounts were made of a few of the eggs stained in borax carmine. These were very useful in orienting one as to the stages seen in sections.

One difficulty was encountered in the preserving fluids. While the chromosomes and cytoplasm, in general, gave beautiful preparations with both preservatives, the little protoplasmic processes did not retain their shape well. This difficulty was never fully overcome as these processes seemed extremely sensitive to both mechanical and chemical stimuli.

As was to be expected from the study of living eggs, a great deal of variation was found in the appearance and behavior of cell elements in the nine different series of eggs studied. The typical behavior of the eggs will be described first and other such variations as are illuminating will be taken up later.

Since a monaster phase is a step in the formation of an amphia-ster, it is very difficult to distinguish true monaster eggs until the nuclear wall has disappeared and the aster has reached considerable size. In the earliest stages the centrosphere of the aster is small and deeply staining and the chromosomes are bunched on one side. This stage is quickly followed by an increase in the size of the centrosphere and a spreading of the chromosomes over one side of the aster (figs. 16, 17). Once reached, this condition persists a long time and corresponds, as M. Boveri has pointed out, to the equatorial plate phase in normal mitosis.

A careful study of these monaster eggs points to two types

being present in all my material,<sup>2</sup> but whether the difference is due to capricious fixation or staining, or is of morphological significance, I am unable to say. Both types of eggs may be found side by side in the same section. In the one type (fig. 16) the center of the centrosphere is more deeply staining, and when one examines the structure under the highest powers, it will be found that this is due to the fact, that the meshes of the reticulum of which the centrosphere is composed, become smaller towards the center (fig. 18). This reticulum in my preparations is continuous with the aster fibers (fig. 18). In monaster eggs of the second type (fig. 17) the net-work of the centrosphere is uniform in size throughout the space so that the whole stains evenly. In other respects, the two types of eggs are alike. The chromosomes lie in a sort of half-shell around the centrosphere and they are always just about the same distance from the edge of the centrosphere (fig. I<sub>1</sub> to I<sub>5</sub> page 472).

As the controls finish the first division and enter into the second cycle, the centrosphere of the monaster eggs slowly increase in size. This carries the rays of the aster, with the chromosomes attached, nearer to the surface of the egg.

About the time half of the controls have divided into four cells, changes begin to appear in the monaster eggs. In part of them the chromosomes, which up to this time have been rod-like, undergo a splitting (fig. 20). This is found in eggs with the clear centrosphere. In the second type of egg, one frequently finds the centriole dividing as is shown in figure 19 and the spindle within the centrosphere may be clearly seen.

After the chromosomes have divided they rapidly approach the edge of the centrosphere, and following this, form little vesicles just as is found in the normal cleavage. At the same time the centrosphere becomes enormously increased in size (figs. 21 and 25) and the rays of the aster reach to the surface

<sup>2</sup> In one series of eggs, I have found, in addition to the two types of monaster eggs, here described, a third which is different and corresponds with the fan-asters ('Flächerkern') of the Hertwigs ('87) and others. Only part of the aster is present and there are only 18 chromosomes present. These eggs are beginning parthenogenetic development, but they soon disintegrate.



of the cell. As the chromosome vesicles fuse to form one nucleus, the rays of the aster appear to break up, (fig. 25) becoming granular, and gradually they entirely disappear. Traces of the lightly staining centrosphere may be found for some time, however. In only a few cases have I found evidence of the retreat of the aster in this type of egg. Two serial sections of such an egg are given in figures 13 and 15. The projections of the protoplasm formed during the movement are here clearly seen.

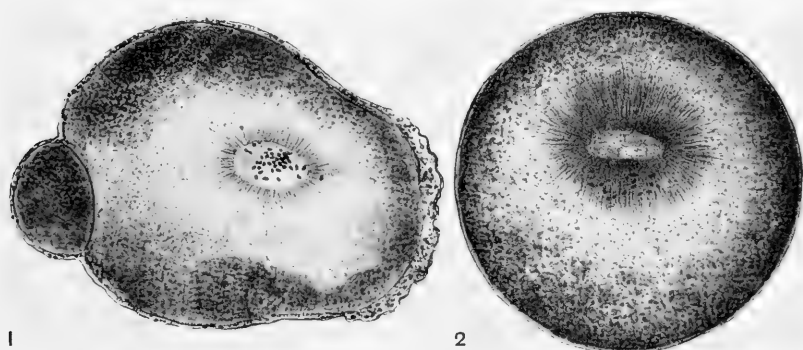


Fig. E

The eggs with a deeply staining centrosphere seem to undergo a different series of changes. Following the division of the centrioles (fig. 19) the centrosphere becomes elongated and moves toward one side of the egg (fig. 19 and text fig. E<sub>2</sub>). After this two things may happen. The centrioles may separate completely and form a spindle between them. This process is usually accompanied by the formation of the peculiar spiral asters which I have described in another place ('16). The determining factor as to whether or not the spindle is formed seems to be the division of the chromosomes. If the chromosomes divide before the centrioles are far apart, then no separation appears to take place, instead nuclear vesicles are formed and these fuse to one nucleus as the astral rays disappear.

Quickly following on the first division cycle, the second one sets in, and the early appearance of the amphiaser is seen from

figures 12 and 14. The spindle which results (fig. 24) appears normal except for the large number of chromosomes lying in the equatorial plate. I have not been able to count the exact number in the spindle, but corresponding stages in monaster eggs show 72 chromosomes (text fig. 9). In telophase stages the asters become extremely large, but I have found no sections through eggs just at the period when cleavage was taking place.

The triasters and tetrasters found in section and observed in the living eggs at this period, are to be derived, I believe, from these monaster eggs in which the centrioles divided early. They do not differ in section from similar eggs produced by dispermy.

Those monaster eggs in which the monaster persists in the second division cycle show considerable variation in appearance. In figure E<sub>1</sub> a rather common form is shown. The swollen ectoplasmic layer, and the irregular shape of the egg are remnants of the protoplasmic movement of the first division cycle. We see here the centrosphere surrounded by very short astral rays to which the chromosomes are attached. Figure 22 shows a later stage when the chromosome vesicles are beginning to fuse.

In figure F<sub>1</sub> to F<sub>7</sub> we have drawings of other types of monaster eggs. Figure F<sub>1</sub> is a later phase of the first monaster cycle. Figure F<sub>2</sub> shows a typical form of the second monaster. A clear defined centrosphere is not to be seen. Instead, the aster rays arise from a ill-defined area, and the chromosomes, instead of lying on one side of the aster, are here scattered all around the aster. Careful counts (fig. G) show about 72 chromosomes in the egg. Not infrequently, instead of one general center, there are four ill-defined centers from which the aster fibers arise. Figures F<sub>4</sub> to F<sub>7</sub> show sections through later stages. The irregular form of the eggs is due to the extraordinary movement of the protoplasm which follows the formation of the nuclear vesicles in the division cycle.

In later stages we may find the persistence of a single aster but more usually a great number are present as is shown in figure 23.

*Discussion*

With his characteristic insight, Boveri realized that the peculiar behavior of the monaster eggs was not to be considered as disintegration phenomena, but that each feature was to be found in normal cleavage. In the short paper already referred to, he calls attention to the main points of similarity in the external behavior of the eggs, and Mrs. Boveri extended the comparison to the cell elements. An exact correspondence was found throughout.

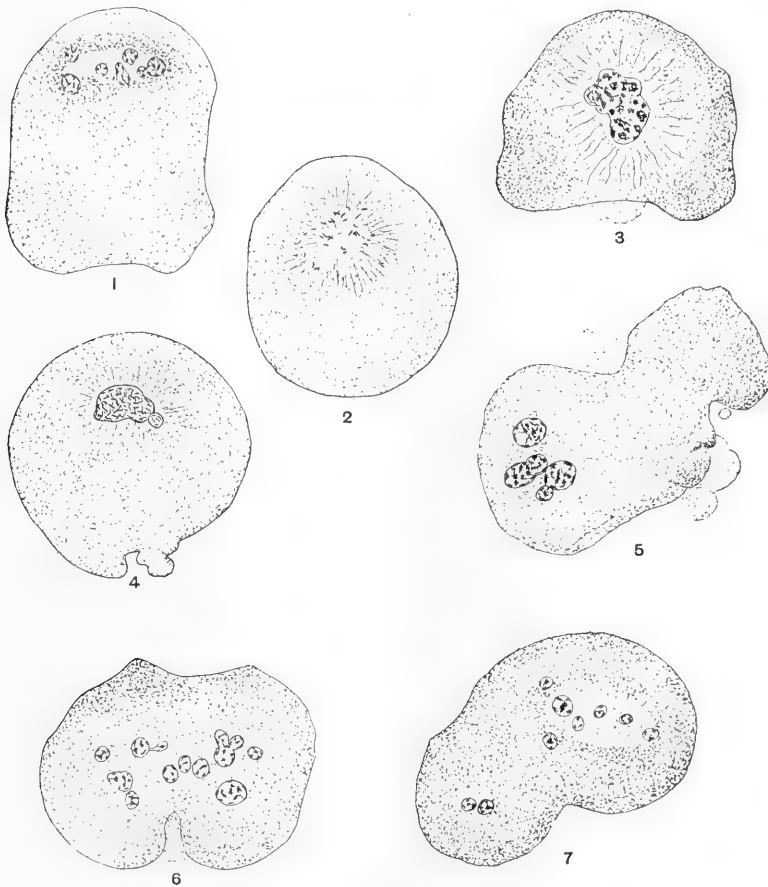


Fig. F

In the living egg, the similarity between normal and monaster eggs may be considered under three heads: the aster, the ectoplasmic or hyaline layer, and the granular cytoplasm.

The appearance of the asters, their growth, their changes in form, and finally their retreat towards the surface of the egg are phenomena so well known for the sea urchin egg that they call for no description here. The single aster passes through the same stages, as my description and figures will show.

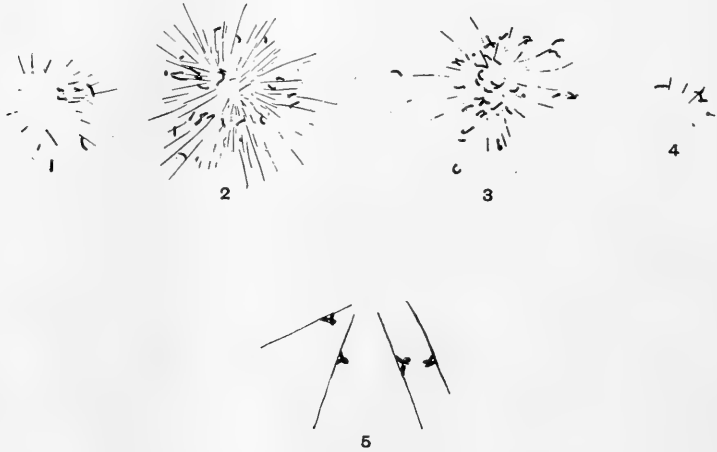


Fig. G

The correspondence in the behavior of the hyaline or ectoplasmic layer calls for a word by way of explanation first, as this part of the egg does not seem to have attracted the attention which it merits. It has been known for a long time (Selenka, '83 was the first to describe it) that there is a thin layer of ectoplasm surrounding the eggs of echinoderms, some molluscs, and ctenophores. This layer lies just beneath the fertilization membrane and, in the sea urchin at least, does not become prominent until the first division cycle is well advanced. It reaches its maximum development over the whole surface of the egg a few minutes before cleavage sets in. Now if one examines eggs with a dim light just as cleavage is about to begin, one will see that part of this layer has become swollen or blistered. This swollen area runs as a narrow girdle around the

equator of the egg and just beneath it the cleavage furrow will appear. This is a very constant feature in *Strongylocentrotus* and has been noted by Boveri, Goldschmidt and Popoff ('08), and many others.

In monaster eggs we have the same swelling or blistering of the ectoplasmic layer, but it appears, not in a girdle between the two asters, but on the whole side of the egg opposite the chromosome side of the aster.

It is the third feature, perhaps, which forms the most striking parallel between the two types of eggs, namely, the behavior of the granular cytoplasm. In normal eggs, just beneath the swollen hyaline layer, the cleavage furrow appears. This gradually separates the egg into two cells, but the walls of the cleavage furrow are not as smooth as they are ordinarily figured. If any egg is observed very closely under moderate powers of the microscope, the furrow walls will be found to be undergoing changes during which little ridges and depressions appear. Finally, as the asters disappear the two new blastomeres round up and all active external movement ceases.

In monaster eggs, following immediately the swelling of the ectoplasmic layer the peculiar movement of the protoplasm sets in, and this leads to changes in surface contour and to the cutting off of protoplasmic masses, both of which indicate that this side of the egg is undergoing intense changes in surface tension. As the astral radiations disappear, these movements become less intense and eventually the normal rounded form of the egg is resumed.

It is clear that the force, or forces, which operate to bring about the separation of the two blastomeres with the attending movement of the furrow walls in normal cleavage are represented in the changes in surface contour and the cutting off of protoplasmic balls in monaster eggs.

In the second and later division cycles one finds in the monaster eggs, at least as far as the ectoplasmic layer and cytoplasm is concerned, the same correspondence to normal cleavage as described above. Thus, in those monaster eggs, which at the

second division cycle shift over into an amphiaster, we find, in the cleavage plane, a swelling of the ectoplasmic layer and changes in surface contour (fig. A) which are similar to, but more severe than, those in normal division. And in those eggs where the monaster persists at the later cycle we have first, the swelling of the hyaline layer, then the movement and then the egg ceases to undergo active changes and resumes its rounded form more or less completely.

We thus see that the behavior of the monaster eggs, as aberrant as it appears at first sight, is similar to what we find in normal cleavage, both in the sequence of events and in the expression of the forces at work. And it gives us a far clearer picture of the intensity of the action of these forces, than that which we see in normal cleavage. No one has doubted that cell division was accompanied by changes in surface tension—any change from the spherical would necessitate this—but the formation of pseudopod-like processes and the general amoeboid movement of the protoplasm, lasting some time and involving a large part of the egg protoplasm, are expressions of a far more powerful force in cell division than normal cleavage would ever lead us to suspect.

One striking feature accompanying the protoplasmic movement, seen best in pigmented eggs, is the movement of the pigment granules (fig. 11). Both in *Strongylocentrotus* and *Arbacia* the pigment lies in the superficial layers of the protoplasm. During the protoplasmic streaming, in the first monaster cycle, most of this pigment tends to collect in the protoplasmic droplets (fig. 11). (This figure is drawn from an *Arbacia* egg). This fact demonstrates that accompanying the movement there is a flow of the cortical layers of protoplasm of the egg towards the side where the surface changes are most intense. This observation is of extreme interest because a number of authors have described a similar flow of substance toward the cleavage plane in other forms, and a good deal of importance has been given to this as a feature of cell division, i.e., Bütschli, and Conklin. A few illustrations will suffice to show how wide-

spread the phenomena is.<sup>3</sup> Nussbaum ('93) observed that the brownish black pigment found in the entodermal and mesodermal cells of young *Rana temporaria*, tended to collect in a ring around the equator of the cell, and with division this pigment sank in and formed a cell plate between the daughter cells. Gardiner ('95) observed substantially the same thing for the cells of *Polychaerus* and *Aphamistoma*. Erlanger ('97) observed in the nematode egg, a movement of the protoplasm first towards the equator, thence inward and thence toward the asters. More recently Conklin ('02 and '05) has described in detail the vortical movements of the protoplasm in *Crepidula*, and *Cynthia*.

5 The intimate connection between the movement of the pigment and the changes in surface tension, during the division cycle of monaster eggs, points either to a common cause, or to the one being the effect of the other. And considering the widespread occurrence of the pigment movement in cells of other forms, it is clear that we are not dealing with a force peculiar to the sea urchin egg, but one common to many, perhaps all dividing cells. Any analysis which throws light on the nature of the surface tension changes, or their causes, is a decided advance.

The first step was to ascertain the cause of the swelling of the ectoplasmic layer. It seemed possible that this was due to some substance diffusing through the egg protoplasm, as Loeb ('95), and Robertson ('08) suggested. If this be true it might be through direct chemical action. I made a few preliminary experiments in Naples with this possibility in mind. Dilute solutions of HCl and NaOH were made in sea water. After the fertilization membrane had been shaken off, the acid was injected against one side of the eggs by means of Barber's apparatus. The ectoplasmic layer increased in thickness. The same results were obtained, however, with the sodium hy-

<sup>3</sup> For a full review of the literature up to 1902 see Conklin's work *Karyokinesis and Cytokinesis*. Since that time I have noted the following works. Fishel ('06) working with *Arbacia pustulosa*. McClendon ('09) working on *Arbacia punctulata*. Harvey ('10) working also with this same species.

dioxide, so I left Naples without reaching any conclusion on this point. Unknown to me then, Goldschmidt and Popoff ('08) had carried out a series of experiments, on sea urchin eggs, bearing on this general question, and their work sets the matter in such a clear light that I will review their paper in some detail.

In the ctenophores, the swelling of the ectoplasmic layer is very conspicuous, and Ziegler ('97) had assigned to it a constricting action in the cleavage of these eggs. This lead Goldschmidt and Popoff to investigate the origin of this layer, and the conditions under which it appears. They found that the layer appeared about the time the pronuclei of the egg were fusing and it steadily increased in thickness as the nucleus prepared for division. Just before cleavage set in, a girdle of swollen ectoplasm marked out the cleavage path.

When the water was allowed to evaporate from the dish in which the eggs were placed, the membrane grew thicker and this lead the authors to suspect that osmotic changes were responsible for its appearance. When eggs were placed in hypertonic sea water as the spindle was being formed, the layer greatly increased in thickness, at the same time numberless little processes of granular cytoplasm penetrated into the transparent layer, anastomosing and branching, giving the layer a vacuolated appearance. As a rule such eggs did not divide, but when a cleavage furrow did appear, the ectoplasmic layer did not sink in with it as it does under normal circumstances. In the space thus left, little radiating fibers (Zuglinien) appeared running from the granular cytoplasm to the layer. Eggs treated with hypertonic sea water showed only an extremely thin layer of ectoplasm in later stages. Also the authors reached the conclusions that the ectoplasmic layer, in later stages, is a true membrane and not an integral part of the egg.

These experiments show that osmotic changes cause the swelling of the ectoplasmic layer, in fact, that an increase in the density of the sea water causes the increase in thickness, while hypotonic sea water tends to inhibit its appearance.

Returning now to the monaster eggs, since the density of the sea water remains the same, we seem forced to conclude that



there is an osmotic change in the protoplasm just beneath that portion of the ectoplasmic layer which swells later. In other words that preceding the active changes of form which the protoplasm undergoes, there are great changes in the osmotic relations of this side of the egg, as is shown by the swelling of the ectoplasmic layer. If we may safely compare the experiments of Goldschmidt and Popoff with our eggs, we should say that the movement was preceded by an increase in the density of one side of the egg.<sup>4</sup> This would carry with it, of course, a difference in the permeability of different parts of the egg.<sup>5</sup>

To what was this change in density due? Three possible sources could be suggested. First the aster, second the nucleus, and third the cytoplasm.

*The aster.* The movement of the aster was first considered as the cause of the movement, because the distance which the aster moved through the egg was a measure of the intensity

<sup>4</sup> This is precisely what Butschli ('92) has maintained, and with him, a number of other investigators. Among the latest of these is McClendon ('10, '12). Robertson ('09, '13) has taken issue with McClendon and claims that, "In cell division the cleavage furrow is a region of low superficial tension, the poles of the egg regions of high superficial tension. Butschli and McClendon's view to the contrary is shown to involve a contradiction of the laws of molecular attraction of the liquids." (Robertson, *Arch. f. Entw. Mech.*, vol. 35, p. 707). Robertson's views are based upon certain experiments with oil drops. "Drops of rancid olive oil were floated upon water, and threads wetted with solutions of strong bases, were laid across the drops. Division resulted owing to the equatorial diminution of surface tension resulting from the formation of soap" (Robertson, *l.c.*, p. 692). In describing the behavior of the oil drops, he distinctly points out that there is a superficial flow of substance away from the equator towards the poles of the egg: "and violent streaming motions occur at the surface away from the thread and towards the opposite poles of the drop." (*Arch. f. Entw. Mech.*, vol. 27, p. 30.) This is exactly the reverse of the condition found in the living egg, by a great number of investigators, including myself. Clearly, the conditions within the egg, are quite different from those in the oil drops with which Robertson experimented. This flow of substance towards the cleavage plane involves an increase of surface tension in this region, as Robertson points out, "for an increase in surface tension at the equator, such as Butschli imagines to occur would result in a streaming of material towards the equator" (p. 29, *Arch. f. Entw. Mech.*, vol. 27). Robertson made the error of comparing the conditions within the oil drop (a homogenous body) with that of the living egg.

<sup>5</sup> See Lillie, '16.

of the movement. Thus, when the aster retreated well up against one side (figs. 2, 3, 4, 5) the movement was very severe, while, when the aster moved little or none at all (fig. B), there was a very slight movement of the protoplasm. It was possible to explain this fact in one of several ways. Later stages, however, did not support the view that the size or movement of the aster played any part, for the simple reason that, while the aster became often smaller and underwent little or no movement (fig.  $F_1$  to  $F_3$ ) the severity of the protoplasmic movement was greater than in the first cycle.

*The nucleus.* The nucleus offered less difficulties as a possible source of the protoplasmic movement than the aster. The protoplasmic movement appears always just as the nuclear vesicles are being formed, and the increase in the number of chromosomes at each succeeding division cycle is accompanied by an increase in the intensity of the movement. Again, the fact that the movement is seen only on that side of the egg towards which the chromosomes are directed (in the first mon-aster cycle) points strongly to the latter as being in some way concerned with the movement. And in latter division cycles when the chromosomes cover the aster more or less completely, the formation of pseudopod-like processes involves nearly the whole egg's surface.

Assuming the nucleus to be the cause of the movement, it would be easy to explain the relation of the aster to it. Cytologists are in general agreement over the fact that the aster tends to exert a solidifying influence over the egg protoplasm, that is, cause the protoplasm within its influence to be less fluid. If this be true then parts of the cell farthest removed from the influence of the aster would be the first to be affected in case some substance was present which altered the surface tension of the protoplasm. In eggs where the aster retreated to one side, the whole opposite side of the egg would be free to move. In case the aster did not retreat, then we should expect to find little movement. Again in eggs, such as monaster eggs of the second or later division cycle, where the aster was small, we should expect to find here the greatest freedom of movement. This corresponds with the observed facts.

On the other hand, three independent workers (Wilson, '01 a, Ziegler '98 a and Mrs. Boveri) have reported that in enucleated blastomeres in which asters were present, the latter have the power of surrounding themselves with a certain amount of protoplasm, and in one case (reported by Ziegler) of actually dividing. While no mention is made here of the swelling of the ectoplasmic layer or of cytoplasmic movement, it seemed possible that both were present. In which case the chromosomes could not be regarded as causing the movement.

*The cytoplasm.* A consideration of the above facts lead me to take up the cytoplasm alone, as causing the movement. The difficulty here was that supposing the swelling of the membrane and the surface tension changes following were the result of certain products produced by the oxidation of the cytoplasm, then we should expect to find that the movement occurred in all the eggs at the same time. This is far from being the case; the egg protoplasm remains unchanged until certain processes are completed in the chromosomes and aster, and this may take place anywhere between the 2- and 8-cell stage of the control.

From the analysis of monaster eggs, it was clear that one could not determine which, of the three possible factors, was responsible for the protoplasmic movement, or whether perhaps all three were involved. Further progress could only be made when one or more of these factors could be eliminated.

An attempt to eliminate the aster from the problem was made by the use of the narcotic, phenyl urethane. The results of the experiments are given below.

#### EXPERIMENTS WITH NARCOTICS

During the course of an experimental study in which the eggs of *Strongylocentrotus* were treated with dilute solutions of the narcotic phenyl urethane, I noticed that eggs left a long time in this solution would finally undergo an irregular sort of cleavage (figs. H<sub>1</sub> to H<sub>4</sub>). Furrows would appear in the egg's surface preceded by a swelling of the ectoplasmic layer, and two or more blastomeres would be cut off. Such divi-

sions were not permanent as a rule. On examining these eggs with high powers I was unable to detect the presence of any radiations in the cytoplasm, although in the central clear area of the egg, a few very faint fibers were visible. A study of the treated eggs was undertaken in order to find out the changes which preceded this cytoplasmic division, with the hope of

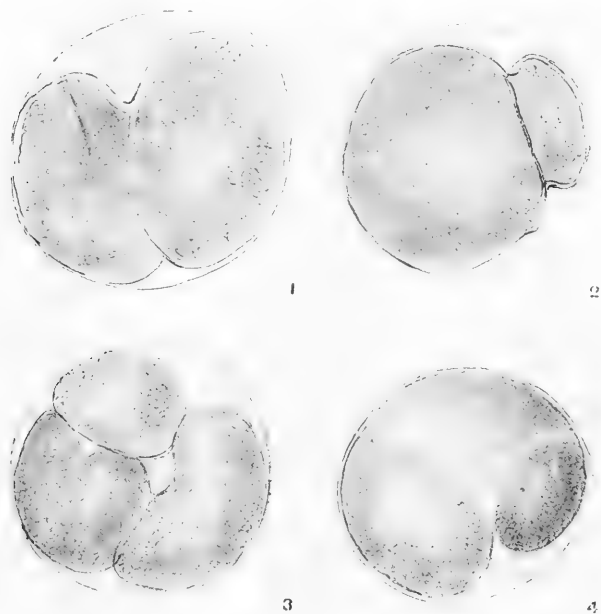


Fig. H

being able to throw light on the cause of the protoplasmic movement in the monaster eggs.

The present experiments were carried on at Woods Hole and the material used was the eggs of *Arbacia punctulata*. After fertilization, the eggs were allowed to undergo development for five to fifteen minutes and then they were placed in a 1/2000 N solution of phenyl urethane in sea water. The eggs were followed under the microscope, and portions both of the controls and of the experimented eggs were preserved at ten minute intervals either in strong Fleming or in sublimate

acetic, since both of these fluids bring out the achromatic structures. Sections were made at  $7\mu$ , the stains used were iron haematoxylin followed by light green.

In the living egg, very little can be made out on account of the heavy pigmentation. In the following description observations from one set of eggs will be given. There was some variation in the different experiments, of course, but the behavior of the eggs was essentially the same in all cases. At the time the eggs were transferred to the narcotic, the male pronucleus was already visible as a very small globular body. After the eggs were placed in the solution, it increased very rapidly in size and slowly approached the female pronucleus. In part of the experiments, an ultimate fusion of the pronuclei took place, in others, the male and female elements remained separate. When the fusion did take place, it was very late, for the controls were in the stage when the aster becomes elongated into a crescent just before a spindle can be seen.

After the fusion of the pronuclei there was a rapid increase in the size of the nucleus due to swelling. In the event that a union did not take place, the male element increased greatly in size while the female pronucleus increased only slightly. Then the nuclear wall disappeared and in the egg one could see that the nuclear area showed traces of fibers. This condition persisted for a long time. At no period did one find cytoplasmic radiation. About the time the controls are in the 4-cell stage, the radiations in the nucleus disappear and following this the cytoplasm begins to undergo a labored division during which two or more blastomeres may be separated. Figures  $H_1$  to  $H_4$  show camera sketches of such eggs made from *in toto* mounts. Accompanying this division there was a decided tendency for the pigment to collect in the division plane, showing that there was a flow of the superficial protoplasm, just as there was in monaster eggs. In some few cases the division was permanent. In most eggs, however, after the cleavage furrows had persisted for some time, they would gradually fade out and the egg would resume its normal rounded form.

In studying the sections the main point to be determined was the condition of the eggs in which this division was taking place, especially was it desirable to see what had become of the aster.

Five minutes after the eggs have been transferred to the solution of the narcotic, one finds the condition shown in figure

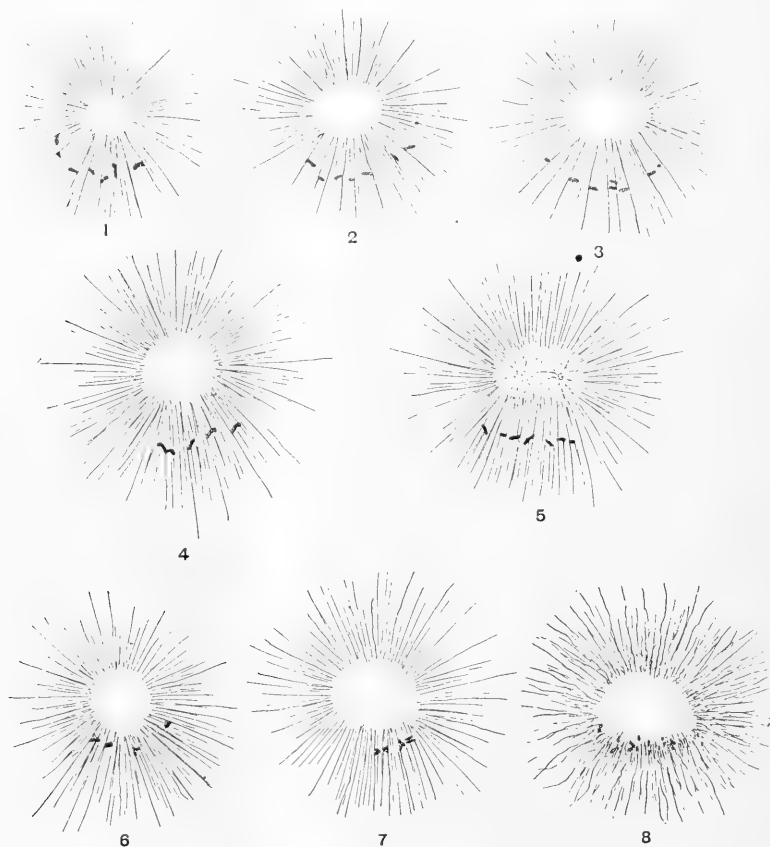


Fig. I

26. The male pronucleus lies at some distance from the female element, it is small and, surrounded in every case in my preparations, by an area which is free from granules. Some sort of fluid seems to have collected around the male element. There is absolutely no sign of any cytoplasmic radiations at this time. Later stages show the approaching and touching of the male

and female pronuclei. It is also interesting to note that the clear fluid area accompanies the male pronucleus up to the time of fusion, but after this it disappears. The swelling following the union of the pronuclei goes on rapidly and the nucleus becomes very large. At the same time, the chromatin collects into definite bodies and just before the nuclear wall disappears the chromosomes show very distinctly, lying on the linin network. As the nuclear wall is dissolving one occasionally sees a centrosome, (fig. 27), which appears to lie on the nuclear wall, and the fibers from this center appear continuous with the linin net-work in which the chromosomes lie. The division of the centrosome seems to already have taken place, for with the disappearance of the nuclear wall, a spindle is formed within the nuclear area (fig. 28). The whole nuclear cavity is filled with exceedingly fine parallel fibers and the chromosomes lie scattered in an irregular equatorial plate. Although I have studied these preparations with the best lenses I have been unable to detect a centrosome at either end of the nuclear area. At this time there are absolutely no cytoplasmic radiations to be found in the eggs of my preparations. Following this an irregular sort of division of the chromosomes seems to take place (fig. 30) and chromosome vesicles form at the two sides of the nuclear area.

In those cases where the pronuclei did not fuse, we find that the nuclear walls disappear at the same time. The chromosomes lie on a network which in some cases has a parallel structure, like that of a spindle (fig. 29). An irregular division of the chromosomes takes place here too and afterwards the chromosome vesicles are formed (fig. 31).

At the time when the nuclear vesicles are fusing into one or more nuclei, we find the movement of the protoplasm beginning. There is the greatest variation in the appearance of the eggs but they all agree in this that the cleavage furrow always cuts through the place occupied by the nucleus (figs. 32 and 33).

The point which I wish to emphasize is this, that at no time during the whole history of these eggs, is there any trace of

cytoplasmic radiations. There is, in short no aster—though centrosomes may be present—and yet there is a flow of protoplasm into the cleavage plane and the eggs divide.

#### FURTHER DISCUSSION

As striking as these experiments with phenyl urethane may appear at first, the results were not wholly unexpected in the light of other researches. Wilson ('01 b) observed that when he allowed the ether to evaporate in the dishes where eggs were, that eventually these eggs would attempt an abortive sort of division.<sup>6</sup> During this time the asters were but feebly developed. And Teichmann ('03), repeating Wilson's experiments on *Echinus microtuberculatus*, remarks that he observed an egg divide after being treated with ether, in which he could observe no astral radiations. Neither of these authors gave the matter any closer study.

In *Arbacia* we have as typical a development of the aster as is to be found among animal cells, and yet the experiments with phenyl urethane show that in the total absence of these striking structures, the egg may undergo cleavage. Whatever the function of the aster may be, these experiments make it clear that a second factor (or set of factors) is at work which operates to bring about cytoplasmic cleavage. This division is accompanied by a swelling of the ectoplasmic layer and a flow of pigment towards the cleavage furrows.

To return to the question of the cause of protoplasmic movement, it is clearly demonstrated that neither the aster nor the processes involved in its formation (centripetal flow of ground substances, Rhumbler ('96), etc.) are responsible for the swelling of the hyaline layer or for the flow of pigment granules into the cleavage furrow or for the cleavage itself. This forms very

<sup>6</sup> Warburg ('11) has shown that dilute solutions of phenyl urethane, such as I have used, do not materially alter the rate of oxidation of sea urchin eggs. Ether, however, (and a number of other narcotics) does diminish the oxidation rate. It is possible that the greater activity which the eggs of my experiment showed was due to this difference.



conclusive evidence that these changes (at least the first two) in monaster eggs are due to another cause.

With the elimination of the aster from our problem, we still have in the nucleus, and in the cytoplasm, two possible sources for the behavior of protoplasm in monaster eggs. I have been unable to devise experiments which would give decisive evidence here. On the whole the mass of evidence is in favor of the nucleus, though we are utterly in the dark as to how it works. In addition to the evidence discussed on page 468 in favor of the nucleus, the experiments with phenyl urethane show that when cytoplasmic cleavage does occur, it cuts through the region where the chromosome spindle lay. The position of the chromosomes seems to play a part here.

As additional evidence we may point out that cytoplasmic cleavage rarely or never occurs in the absence of chromatin from the spindle. This is nowhere shown with greater clearness than in Boveri's paper "*Zur Physiologie der Kern-und Zellteilung*" ('97). In cells with tetrasters, cleavage only took place between poles in which chromosomes were present. Although shallow cleavage furrows might appear between asters where no chromatin was present, lasting division was never seen.

While at first sight the elaboration of Loeb's suggestion which Robertson ('08) has given seems plausible enough, there are difficulties to be met which are not easy to overcome. As is well known, Robertson suggested that some substance (cholin) diffused through the cell cytoplasm on the disappearance of the nuclear wall, and when this substance reached the surface of the egg, it formed soaps which lowered the surface tension of this part of the egg and hence brought about division.<sup>7</sup> If the swelling of the ectoplasmic layer and the phenomena following it were the result of simple diffusion, it seems probable that it would appear in all eggs about the same time. Far from this, as I have repeatedly pointed out, the period when the

<sup>7</sup> Concerning Robertson's contention that cell division is brought about by a lowering of the surface tension at the equator of the egg, see footnote 4, page 467.

movement occurs is extremely variable and depends apparently on certain changes in the aster and chromosomes. If monaster eggs alone were to be considered, this objection might be met by assuming that the aster exercised a retarding influence on diffusion, but in those eggs treated with phenyl methane, in the absence of asters, the division of the egg was retarded until after the chromosomes had split.

Further speculation of the subject is not desirable until we have more data upon which to go. The one outstanding fact is that there is a second factor entering into the cleavage of the sea urchin egg, a factor (or set of factors) characterized by a swelling of the ectoplasmic layer, great changes in the surface tension of the protoplasm, and a flow of the pigment towards the cleavage plane.

Is this second factor, which the monaster and narcotized eggs lead us to assume, something new? The answer is clearly in the negative. The monaster and narcotized eggs only allow us to see far more clearly the expression of this second force than normal eggs do, and furthermore, permit us to harmonize the widely differing views of other investigators.

As long ago as 1876 Butschli gave a mechanical theory of cell division based on movements of the protoplasm, and since that time a number of authors have observed movements towards the cleavage plane, which might be of significance in cell division. Most conspicuous of these are the observations of Erlanger ('97) who studied living nematod eggs at Butschli's suggestion, (see Butschli '00) and the works of Conklin, who finds in "the vortical flow of cell substance" (Karyokinesis and Cytokinesis, p. 100) one of the most important factors in cell division.

From a different standpoint we have Loeb's ('95) suggestion:

Ich stelle mir nun vor, dass an der Oberfläche des Eies, möglicherweise in demjenigen Meridian oder Kreise, dessen Ebene die beiden Strahlensysteme der Centrosome von einander trennt, sobald die Kerntheilung physikalisch abgelaufen ist, Ausbreitungserscheinungen stattfinden. Dieselben führen zur Bildung von in Bezug auf diese Ebene symmetrischen Wirbelbewegungen.

Rhumbler ('96) finds in the growth of the membrane (*gesteigertes Membranwachstum*) a factor which assists the asters in cell division. This phase of cell division is more fully elaborated in a later paper (Rhumbler '99).

Zeigler ('98 b) interpreted the swelling of the ectoplasmic layer in ctenophores in a different way, as I pointed out on page 466.

More recently we have works which approach the problem from the standpoint of physical chemistry. Foremost among these is Lillie's work, who finds that there is a distinct change in the permeability of the cell during each division cycle, and as he points out, this would be accompanied by changes in surface tension (Lillie '16).

A moment's consideration will show that these three widely differing views on cell division may be united by this study of monaster eggs. The swelling of the ectoplasmic layer is just the same phenomena described by Rhumbler and Zeigler. No where is the "vortical flow of cell substance" more clearly seen than in monaster eggs. And finally the great changes in surface tension which monaster eggs undergo indicate a difference in density (and of necessity permeability) on the several sides of the egg. In monaster eggs, however, these phenomena follow each other in regular order, and appear to be due either to a single factor, or several factors working together. Perhaps the whole series of events may be traced to a difference in the density of the protoplasm on the side of the egg opposite the chromosomes in monaster eggs, or to a dense girdle of protoplasm in the case of normal eggs. Later researches must throw light here. But a distinct advance has been made, I believe, when we are able to harmonize the discordant views of so many investigators, and show, as I think the above analysis does, that they were all dealing with different expressions of the same force.

## THE CENTROSOME

The present work throws no new light upon the origin and nature of the centrosome, but certain phases in the behavior of the division mechanism call for special mention here.

Normally, the monaster condition lasts for a very short time in the division cycle, only so long as the centrioles are undivided, according to Boveri (*Zellenstudien* IV). In monaster eggs this period may be prolonged through a whole division cycle without seriously disturbing the division mechanism. We are totally unable to suggest the cause of this delay in this division (or the separation of the centrioles), but it seems clear that the abnormal condition may be overcome at any period in the division cycle. Thus a few eggs recover before the controls have divided. In others the centrioles divide and separate when the controls are preparing for the second division. In others the whole division cycle may be passed through, and this is what occurs in the majority of the eggs. Or finally the separation may be suppressed until the third or fourth division cycle has been passed through; or, even permanently.

The suppression of one division does not affect the centrioles injuriously, in the majority of the eggs, but frequently in eggs which have passed through several cycles in the monocentric condition polyasters suddenly appear. How are we to explain such cases? Two possibilities present themselves. If we assume that centrioles be at the focal point of the rays, either the centrioles have divided at each division cycle but have failed to separate, a view for which I have no evidence to support, or, as the inhibiting factor is removed, the centriole have been excited to rapid division. The second effect may be experimentally obtained by treating eggs with various poisons.

Another feature which should be mentioned is the increased size of the asters in those eggs which have passed through one division cycle and have then formed amphiasters. The appended figures  $K_1$  and  $K_2$  show the size of the normal asters  $K_2$  (in the first division) and the spindle of an egg with a monaster history  $K_1$ . The difference in size at the same phase of

development will be apparent on the inspection of the figures. Boveri along with a number of other investigators has pointed out that there is a constant relation between the size of the aster and the protoplasm it controls. That is, a monaster would be larger than a single aster of an amphiaster were the egg the same size in both cases. These spindles with a monaster history form an exception to this rule. Again we are confronted with two possible explanations. If we adhere to the old view of a definite archoplasmic substance, then we can say that while

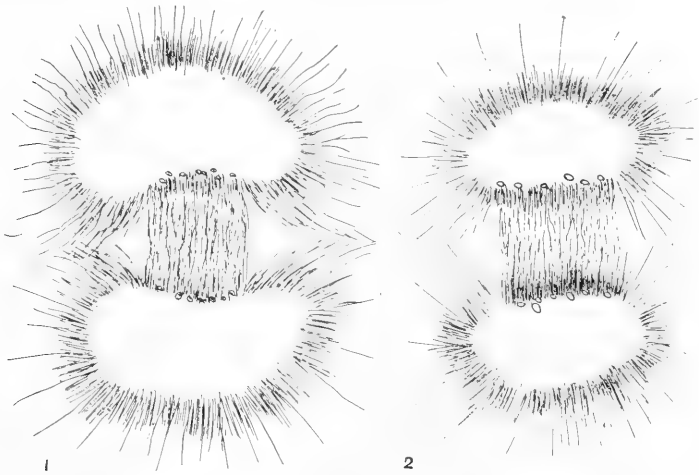


Fig. K

the centrioles may have failed to divide, this substance has increased in amount. Hence at the second division cycle, the asters are larger. The more likely explanation, however, seems to me to be in the relative amounts of chromatin, in these eggs. A number of investigators have maintained that the increase in the size of the asters is due, in part at least, to the absorption of nuclear sap, which is set free in the egg cytoplasm when the nuclear wall disappears. In these eggs with larger asters, we have just double the number of chromosomes that are found in the first spindle. If the nucleus contributes to the growth of the aster, the large size of the asters in eggs with a monaster history is not difficult to explain.

## THE ASTER AND CYTOPLASMIC DIVISION

The centrosome with its surrounding aster has been assigned such a prominent rôle in cell division by most investigators working on animal cells that it is very desirable to arrive at some conclusion as to its function on the basis of the present work. At first sight the present investigation seems to minimize the importance of the aster in cell division. Indeed, I believe that it has been clearly shown, especially by the narcotized eggs, that in the total absence of the asters, the eggs may divide. To conclude from this that the asters do not have a rôle, and a very important one, would be a very serious mistake. For the narcotized eggs and, particularly the monaster eggs, demonstrate that while a force, independent of the aster, is at work within the cell, this force produces little more than striking changes in surface tension which do not accomplish any definite regular result. A directive structure is lacking in monaster and narcotized eggs, and this fact I believe, points out to us the principal function of the aster in the sea urchin egg.<sup>8</sup> A number of facts point to the correctness of this view.

First of all, the work on enucleated blastomeres in which asters are present show clearly that each aster has the power of surrounding itself with a certain amount of cytoplasm. When these asters are near the surface, furrows may appear around or between them, a phenomenon which can only be understood when we assume that the area around each aster is more solid than the outlying cytoplasm. This gives us the necessary mechanism for the directing of this second force which causes surface tension changes.

Again the variation in the intensity of the protoplasmic movement in the first monaster cycle can best be explained by assuming that the aster acts as a center of more solid protoplasm. Thus when the aster undergoes no retreat (fig. B<sub>2</sub>) the force which produces the movement does little more than cause the ectoplasmic layer to swell, since the protoplasm is held fast until after the critical period has passed. Figure D<sub>2</sub> shows an

<sup>8</sup> This applied to cytoplasmic division.

egg where the aster retreated part way to the surface. Here the protoplasm released from its control is free to undergo a certain amount of movement and finally, in those commonest cases in which the aster moved until it lay well up against one side (fig. 1, etc.) the movement was most severe.

Undoubtedly the severe movement of the protoplasm during the change of monaster eggs (figs.  $A_1$  and  $A_2$ ) is due to the same cause. As I have pointed out, the spindle usually lies somewhat excentrically in these eggs with the result that the side farthest removed from the spindle shows the greatest amount of surface tension change. And in those eggs in which the monaster appears at the second division cycle, the severe movement, while due, perhaps to some extent to the increased number of chromosomes, is also to be correlated with the feeble development of the astral system.

These facts taken together with the evidence from the narcotized eggs point unmistakably to the asters as regulative centers during cell division centers, which determine the channels in which the second factor will operate.

#### BEHAVIOR OF THE CHROMOSOMES

A number of authors (Boveri, M. Boveri, Wilson et al.) have pointed out that monaster eggs demonstrate that the chromosomes do not require two opposing centers to pull them apart in order for them to divide and separate; the process is autonomous. A close study of the behavior of these bodies reveals several points of interest.

When the chromosomes become scattered over one side of the aster, they all come to be at about the same distance from the edge of the centrosphere (fig.  $H_1$ ) and are attached to it by means of fibers. The relation is established very early, and, corresponds to the equatorial plate phase of normal division. Following this, the aster undergoes a slow increase in size (figs.  $H_2$  to  $H_4$ ) which causes the edge of the centrosphere to approach nearer to the periphery of the egg. The chromosomes are carried along on their fibers, so that they come to be farther and farther from the middle of the centrosphere. A close

inspection of figures  $H_1$  and  $H_5$  will show that in all these cases the distance between the edge of the centrospheres and the chromosomes remains about the same.

This relation between the chromosomes and the edge of the centrospheres is retained until the chromosomes begin to split. As soon as division begins, the chromosomes rapidly approach the edge of the centrosphere (figs.  $H_6$  to  $H_8$ ).

A close study of the dividing chromosomes shows that the splitting takes place in one of two ways. Sometimes we find a clear line appearing between the two halves (fig.  $G_2$ ) which separate more as they approach the centrosphere. More usually, however, the splitting is accompanied by a twisting of the two halves (fig.  $G_2$ ) about each other and the formation of V-shaped figures. In either case, both halves retain their attachment to the same fiber, and this condition persists until the chromosomes have reached the edge of the centrosphere, in the vast majority of cases. Here and there, however, the two halves appear to be attached to different fibers, as they approach the sphere.

While no light can be thrown on the cause of the chromosome division, it should be pointed out that it appears entirely independent of the condition of the aster. The latter may be large or small, round or sunken in, or the centrioles may even be undergoing division. The chromosomes and the asters seem totally independent in their behavior.

By carefully measuring the distances between the chromosomes and the edge of the centrosphere, I have found that this is approximately the same in all monaster eggs in which the chromosomes are undivided. There is no sign of any movement of the chromosome until a split appears. Then the approach to the edge of the centrosphere is very rapid and few intermediate stages can be found. The division of the chromosomes seems to give the necessary stimulus which causes them to approach to the centrosphere.

This fact that no movement of the chromosomes takes place until after they have divided has an interesting bearing on the question of the forces at work here. As is well known, a num-



ber of theories have been advanced to account for the chromosome movement. Of these, three may be considered the more important. (1) Contractibility of fibrillae. Klein ('79), Van Beneden ('83), Boveri ('88), etc. (2) A pushing apart of the chromosomes; Growth of central spindle, Drüner ('95) etc., or Wataase's ('93) ingenious suggestion. (3) Possibly carried along by protoplasmic movements, Bütschli ('76), or by chemotaxis Strasburger ('93). Most of the later writers on the subject have been inclined to Bütschli's view. Wilson ('95), Griffith ('99), Conklin ('02 and '05).<sup>9</sup>

Of the many objections to the contractile hypothesis, that urged by Wilson, and by Griffith seems the most cogent. In sea urchin eggs after the chromosomes have come to lie against the edge of the centrosphere, they spread out over its surface, a fact irreconcilable with the contraction theory. In monaster eggs this spreading of the chromosomes is seen exceptionally well.

Any hypothesis which attributes the movement of the chromosomes to interzonal fibers, can not explain the monaster eggs, since no such structures are present. Yet the chromosomes approach the centrosphere with rapidity and certainty.

This study of monaster eggs has brought to light no new facts inconsistent with the view that the chromosomes are carried to the aster by the flow of protoplasm. For as the chromosomes divide, the aster begins a rapid increase in size. It is interesting to note that no matter how long the chromosomes remain undivided, no great increase of the aster occurs until they are cleaving. There is a close connection here between the two phenomena.

<sup>9</sup> The question of chromosome movement, which was so much discussed in the nineties, has recently received little attention. For a full review of the literature of this earlier period see Wilson's 'The Cell,' pp. 70-77, also Conklin's Karyokinesis and Cytokinesis. Although written some twenty years ago, Wilson's remarks still seem pertinent: "In any case, I believe that no satisfactory hypothesis can be framed that does not reckon with the chemical and physical changes going on in the centrosphere, and take into account also the probability of a dynamic action radiating from it into the surrounding structures." ('The Cell' page 77.) Such a theory has yet to be advanced, but several attempts have been made in this direction. See Lillie's various studies on Cell Division. Also Hartog ('14).

## 'SPINNING' OR 'FILOSE ACTIVITIES' IN THE LIGHT OF MONASTER EGGS

The protoplasmic movement of monaster eggs is similar, in several respects, to the 'spin-thread', or 'filose activities' observed by several investigators (G. F. Andrews '97, E. A. Andrews '98, Coe '99, C. B. Wilson, '99) in the polar body formation and in the cleavage of nemertean eggs. Also in starfish and sea urchin eggs as described by Mrs. Andrews.

As far as the protoplasmic activity of *Cerebratulus* is concerned, I believe the movements so well described by C. B. Wilson, and which I have myself observed on this same material, may be directly compared to those found in monaster eggs, since: first, they are found in the region of the cleavage furrow; and, second, are found just when cytoplasmic division is taking place, or has just been completed. So far as I am aware, nothing comparable to the swelling of the ectoplasmic layer has been described for the nemertean egg.

The 'filose activities,' described by Mrs. Andrews, were not limited to the division plane, but occurred at any place on the eggs surface and at any time in the division cycle. What relation these general activities bear to the periodic movements, described in this paper, is not altogether clear. The work of Goldschmidt and Popoff suggests a possible explanation. They observed that when eggs were placed in hypertonic sea water, the protoplasm lying just beneath hyaline layers underwent changes in form, during which little threads were formed; these branched and anastomosed, just as Mrs. Andrews described. Under the conditions which Mrs. Andrews' work was made, it seems very likely that an evaporation of sea water took place, with the consequent increase in the density of the sea water. This may have been the primary cause for the activities which Mrs. Andrews observed. In this event, the periodic movements during certain phases of the division cycle, and the continuous activities, would be similar in so far as they are responses of the cytoplasm to the same stimulus, namely, changes in density. In the one case, however, the changes come from within (monaster eggs), in the other, from without.

## SUMMARY

1. A study of monaster eggs, living and in sections, has been made.

2. It has been shown that, at each division cycle, the egg protoplasm is affected in three ways: (a) first there is a pronounced swelling of the ectoplasmic layer, (b) this is followed by intense changes in surface tension leading to the formation of pseudopod-like processes, (c) and accompanied by a flow of the superficial protoplasm toward the area where the streaming is taking place.

3. The intensity of the protoplasmic movement varies with the position which the aster takes at the end of the division cycle.

4. A comparison shows that the behavior of monaster and normal sea urchin eggs is similar.

5. The changes in the egg protoplasm are preceded by definite changes in the aster and in the chromosomes, and an analysis of the behavior of the eggs is made to determine whether these phenomena are due to the aster, nucleus, protoplasm, or to a combination of these.

6. No decisive evidence can be given to prove which part of the cell is concerned, but many facts point to the nucleus.

7. The swelling of the ectoplasmic layer may be produced by treating eggs with hypertonic sea water.

8. An attempt is made to eliminate the aster as a possible source for the behavior of the monaster egg, by treating eggs with phenyl urethane.

9. The experiments show that, in the absence of asters, the eggs may divide, the division being accompanied, in some cases, by a swelling of the ectoplasmic layer in the cleavage plane and a movement of the protoplasm into the cleavage furrow.

10. These facts eliminate the aster (and the processes which presumably lead to their formation, centripetal flow of protoplasm, etc.) from the problem.

11. All evidence points to the nucleus as the source of a second factor in cell division, a factor (or factors) which finds its expression in, a swelling of the ectoplasm, great changes in

surface tension and a flow of the cortical layers of protoplasm toward the cleavage area.

12. It is pointed out that this nuclear factor is not a new conception, but that its various expressions have been emphasized in turn, by a number of different investigators, working on different forms.

13. Evidence has been given to show that, as far as the cytoplasm is concerned, the asters play the rôle of regulative centers during cell division, that is, by forming denser areas they restrict the action of the nuclear factor to a prescribed area.

14. The history of the chromosomes is followed.

15. It is suggested that the 'spinning activities' or 'filose activities' of *Cerebratulus* eggs is similar to the protoplasmic movement of monaster eggs. In the case of the 'filose activities' described by Mrs. Andrews it seems possible that they are responses of the irritable protoplasm to a stimulus similar to that in the monaster eggs, but coming from a different source.

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## EXPLANATION OF PLATES

Owing to the fact that this study has been made at different places, the drawings are not all of the same magnification. All the drawings up to and including figure 26, plate 4, were made, or completed, by Miss L. Krause of the Osborn Zoological Laboratory, Yale University, New Haven, Conn.

## PLATE 1

## EXPLANATION OF FIGURES

All drawings, with exception of 1 and 2, are from free hand sketches of *Strongylocentrotus* eggs.

1. Camera sketch of egg showing swelling of the hyaline layer.
- 2 to 5 Various stages of the protoplasmic movement in monaster eggs. Figure 2 is from a camera drawing.
- 6 Protoplasmic movement of second division cycle.
- 7 and 8 Dividing monaster eggs.
- 9 Disintegrating monaster egg.

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## PLATE 2

### EXPLANATION OF FIGURES

All figures are from *Strongylocentrotus* except 11. All drawings were made with a camera lucida except 10 which is from a free hand sketch. Magnification varies as noted below.

10 Protoplasmic movement in later division cycle.  $\times 1100$ .

11 Protoplasmic movement in *Arbacia* egg showing migration of pigment.  $\times 1200$ .

12 and 14 Monaster eggs beginning second division cycle.  $\times 1200$ .

13 and 15 Consecutive sections through a monaster egg during the protoplasmic movement.  $\times 1000$ .



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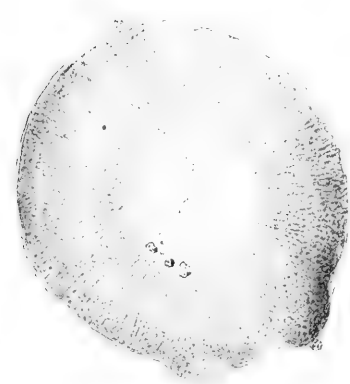


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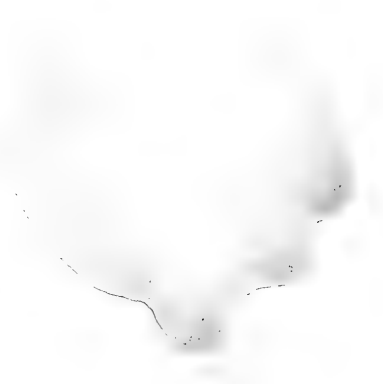
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### PLATE 3

#### EXPLANATION OF FIGURES

Camera drawings of sections. Magnification same as noted below. All eggs are from *Strongylocentrotus*.

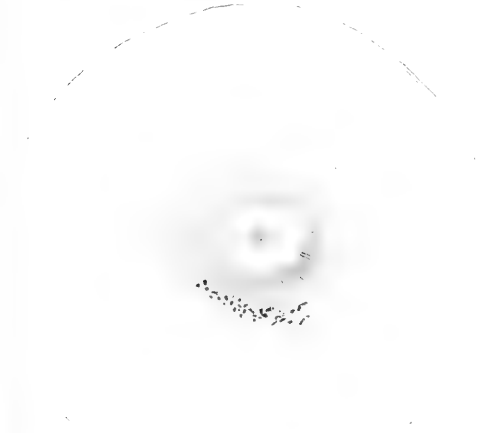
16 and 17 showing the two types of monaster eggs found during the first division cycle.  $\times 1900$ .

18 Detail drawing of aster.  $\times$  approximately 3000.

19 Monaster egg showing division of the centrioles.  $\times 1500$ .

20 Monaster egg showing division of the chromosome.  $\times 1900$ .

21 Monaster egg showing fusion of chromosome vesicle.  $\times 1500$ .



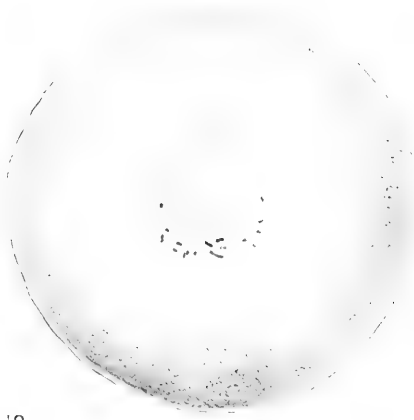
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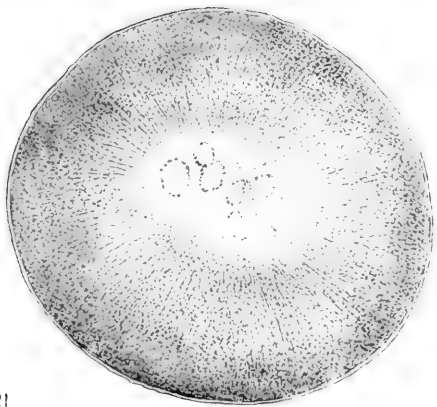
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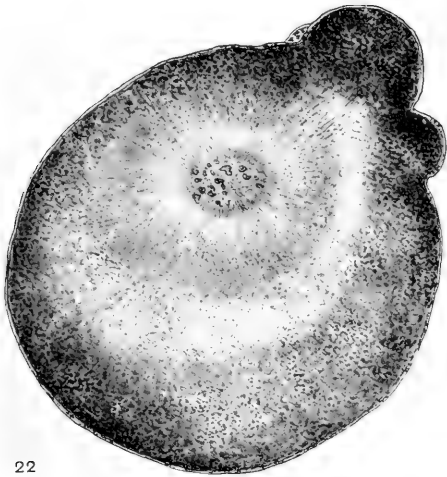
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## PLATE 4

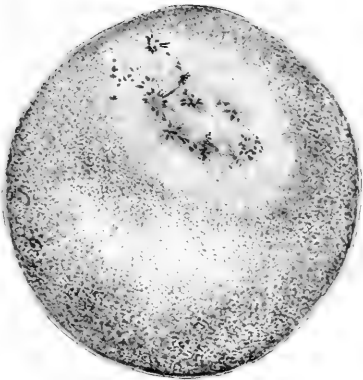
### EXPLANATION OF FIGURES

Figures 26 and 27 from *Arbacia*. The rest from *Strongylocentrotus*. All drawings made by means of a camera lucida.

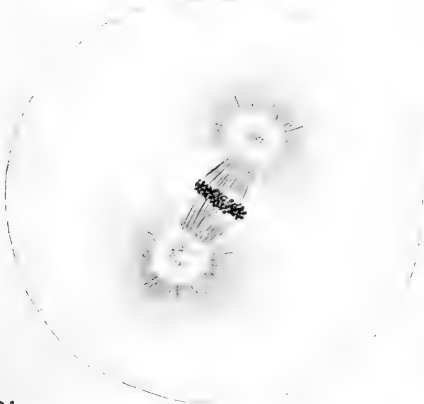
- 22 Monaster egg in later division cycle.  $\times 2100$ .
- 23 Monaster egg showing multiple asters.  $\times 1600$ .
- 24 Spindle in second division cycle of monaster egg.  $\times 1900$ .
- 25 Monaster egg showing chromosome vesicles.  $\times 2100$ .
- 26 *Arbacia* egg treated with a narcotic showing pronuclei.  $\times 2100$ .
- 27 *Arbacia* egg treated with a narcotic showing centrosome.  $\times 2100$ .



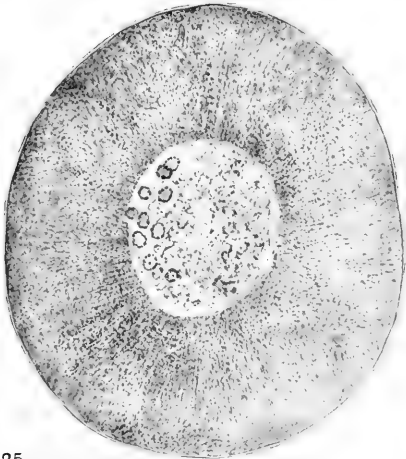
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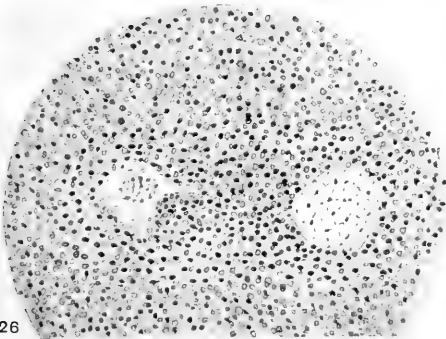
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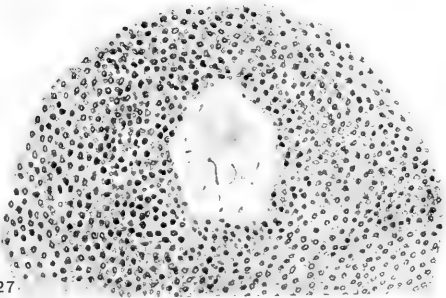
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## PLATE 5

### EXPLANATION OF FIGURES

Drawings from sections of Arbacia eggs treated with narcotic, made with the aid of a camera lucida. Magnification approximately  $\times 2100$ .

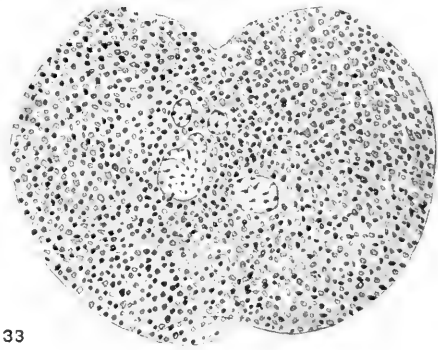
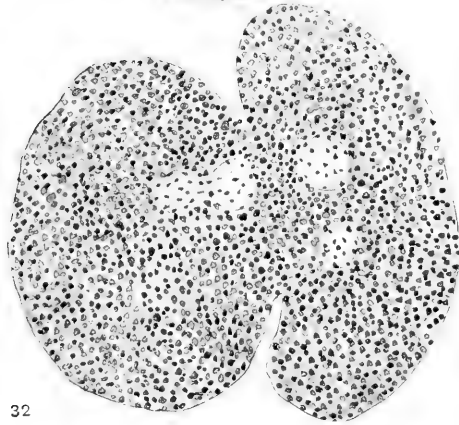
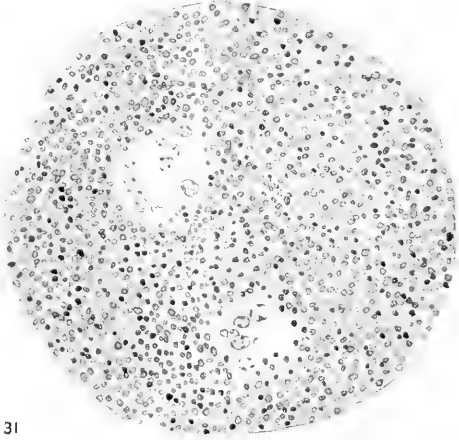
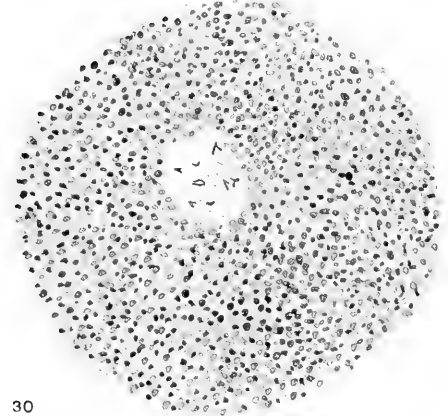
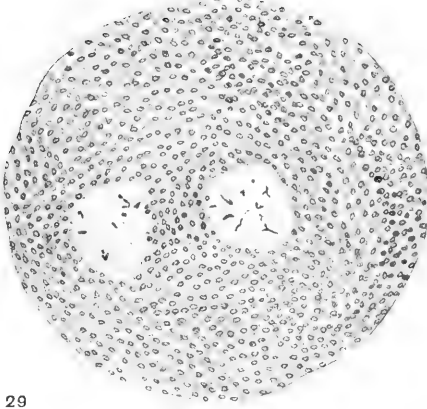
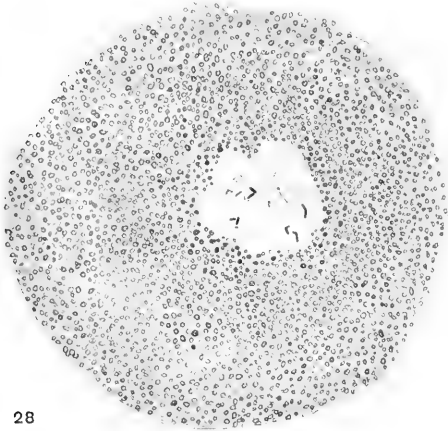
28 Egg showing nuclear spindle with absence of asters.

29 Egg in which the pronuclei did not fuse before the nucleus was dissolved.

30 Egg showing the division of the chromosome.

31 Egg showing the formation of chromosome vesicles.

32 and 33 Eggs showing cytoplasmic cleavage in narcotized eggs.







## THE RESULTS OF THYROID REMOVAL IN THE LARVAE OF RANA PIPIENS

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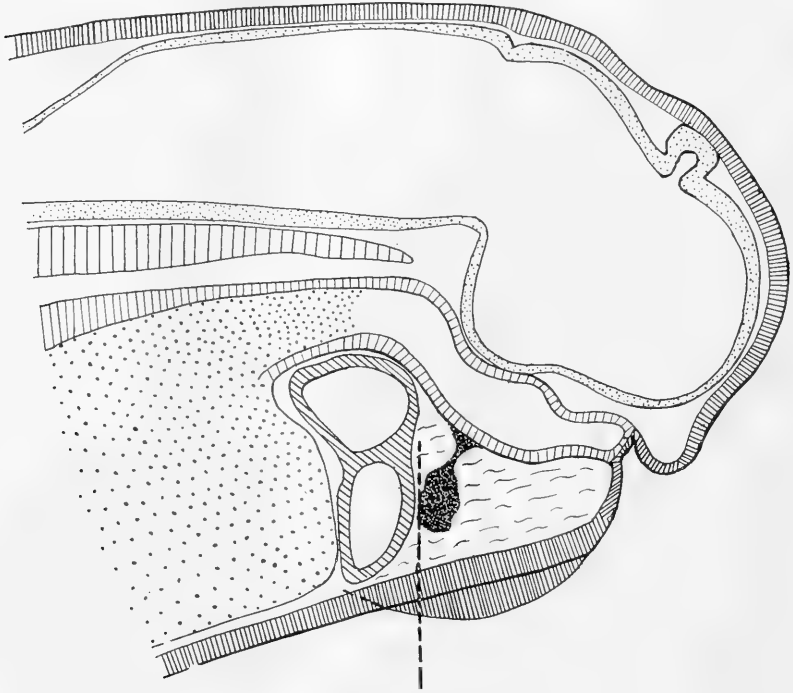
EIGHT TEXT FIGURES AND ONE PLATE

There have been many experiments upon the effects of removal of the thyroid gland in the adults and young of mammals. Much has been learned regarding its effect upon metabolism and upon growth and we have multitudinous clinical data upon thyroid insufficiency; but up to the present time there have been no accounts of the effects of the development of animals from which the thyroid gland has been removed at the very beginning of its development. It is obviously extremely difficult to accomplish this operation in the early embryonic stages of reptiles and birds, while in the mammals such an operation would appear to be well nigh impossible. Because of these difficulties of technique we must turn to the amphibians for such experiments.

The work of Gudernatsch ('12 and '14) many times verified by others showed clearly that thyroid feeding (giving an excess of thyroid secretion) greatly accelerates the differentiation—metamorphosis of tadpoles, causing that process to take place very precociously thus producing absurdly small frogs with all four limbs, with no tail, with the frog type of head and with an alimentary tract greatly reduced in length as compared with controls of the same age.

The writer thought that the complete extirpation of the thyroid gland might give equally striking evidence of the influence exerted by that gland upon development. This hypothesis was most thoroughly vindicated by the results of these experiments. This account will deal with the general effects upon the body as a whole and in a more special way with the effects upon the

development of the gonads and germ cells. Two of my graduate students are working upon the effects of this operation upon other organs. Mr. George Terry is studying the effects upon the skeleton and Mr. James Rogers is investigating the effects of its removal upon the other glands of internal secretion.



Text fig. 1 Diagram of a sagittal section of the tadpole at the stage when the operation is carried out. This shows the position of the thyroid anlage. The dotted line immediately behind it shows the plane in which the cut is made.

The thyroid anlage was removed at its very inception in tadpoles of 6 to 7 mm. total length. This was done by making a transverse cut between the thyroid anlage and the pericardial cavity as shown in text figure 1. With some practice it was possible to do this quite accurately. The cut was quite deep usually laying open the ventral portion of the pharynx cavity. The thyroid anlage is pigmented and with some practice it can be recognized with great precision. As results will show, there

was occasional failure to remove the thyroid anlage. These cases were probably due to failure to remove all of the gland when it was cut in two as frequently occurred. Whenever this was noticed both portions were removed. Care was usually taken to remove the portions of the pharynx floor adjacent to the point from which the thyroid anlage was growing. In some instances there might have been a renewed growth of the anlage from the point of origin.

It may be well to point out that one can always determine the presence or absence of the thyroid gland in later stages preserved for study. This was done by a painstaking study of sections of the lower jaw back to and including the anterior portion of the heart in all specimens used to establish cardinal points in this work. The epithelial bodies (parathyroid glands) were not removed, nor was there any evidence that the results detailed below were secondarily caused as a result of failure of other glands of internal secretion to develop to full functional capacity.

The thyroid anlage was removed with a cataract needle. Within half an hour the wound had completely healed and the tadpoles appeared to be quite normal.

It may be pointed out that the mortality which decimated the half grown tadpoles was due to failure to care for the specimens properly, since the controls showed just as high a mortality as did the thyroidless tadpoles. In spite of the loss of specimens due to failure to provide ideal conditions for them, a sufficient number were retained for firmly establishing the points set forth. At this date, June 10, 1917 three thyroidless tadpoles are still alive—considerably over a year after the operation, and twelve have been killed at various dates from September 20 to the present time. Others were preserved at frequent intervals between the time of operation and that date, so it will be seen that there is an ample amount of material at hand.

The first lot of tadpoles were operated upon April 2, 1916 and the last lot on April 20 as follows:

EXPERIMENT NUMBER	DATE	NUMBER OPERATED
6	April 2	25
7	April 3	15
8	April 4	5
12	April 8	40
15	April 11	69
16	April 13	53
20	April 15	142
Total.....		349

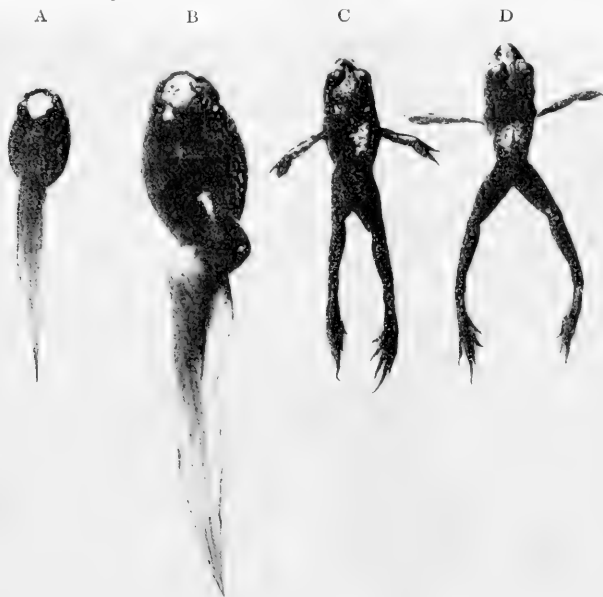
The earlier experiments were not maintained for long, many of the tadpoles being preserved for study in early stages. Lots 15, 16 and 20 however, were kept for a long time and from them were reared those later stages which truly form a basis for this work.

It was quite impossible to foresee what the results of this experiment might be. Early results were expected and eagerly searched for but no peculiarities attributable to thyroid removal were observed until about June 20 eleven weeks after the operation had been performed.

In lot 15 eight died inside of 2 hours after the operation, but this was the only case where any appreciable mortality resulted from the operation. No others died for over a month. Lots 15, 16, and 20 were the most carefully kept. In lot 16 the first mortality occurred on May 20, 37 days after the operation. In lot 20 the first mortality occurred on May 20, 26 days after the operation, from this time on a few died from time to time until June 10, when there was a rapid acceleration of mortality. This affected the controls in slightly greater degree than the thyroidless tadpoles and was not in any degree whatever to be accounted as a result of the operation. This mortality of thyroidless tadpoles and controls was probably due to the fact that they had increased in size so that they had outgrown their quarters. They were kept in aquaria consisting of a series of kitchen sinks each with about 5 inches of water replenished with fresh water drip-

ping from a tap. The Lawrence city water is very hard with 584 mg. of solids per litre.

On June 10 both the thyroidless tadpoles and the controls having reached a length of 35 to 40 mm. showed further unfavorable effects that I have attributed to the quality of the city water. This consisted in a lateral twisting of the tail that constituted a marked deformity. It should be kept in mind that this was



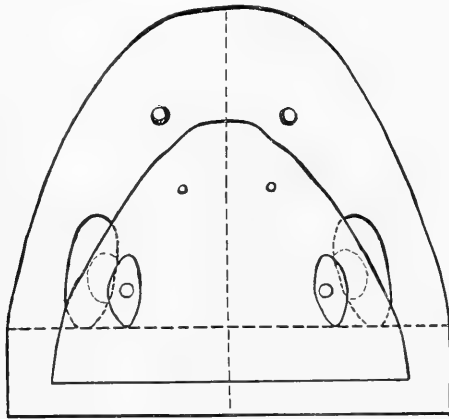
Text fig. 2 Photograph taken August 6. *A*, Undersized thyroidless tadpole; *B*, large thyroidless tadpole with body length of 34.5 mm. The twist of the tail is abnormal. It is discussed in the text. *C*, a metamorphosed control; *D*, metamorphosed operated tadpole. Section of the head showed that there had been incomplete removal of the thyroid gland. A sufficiently large thyroid gland had developed to produce metamorphosis.

equally true in both the operated and the control specimens and hence was not in any sense attributable to the absence of the thyroid. It appeared at the time when the mortality was greatest. I have never seen such distortions of the tail in the case of tadpoles kept in a normal water supply.

This modification affected only the tail and did not in any degree hinder the metamorphosis of the controls, although many of them showed extreme distortion of the tail.

In my experiments upon removal of the anterior lobe anlage of the hypophysis of the tadpole (Allen, Biol. Bull., March, '17) it was shown that the operation had an extremely unfavorable influence upon the tadpoles. I have advanced the suggestion that the hypophysis plays an important rôle in metabolism even during these early stages, and that in its absence the tadpole is unable to cope with the heavy mineral content of the water.

Nothing of this kind was seen in the early development of the tadpoles deprived of the thyroid gland but still retaining the hypophysis. They in no wise differed from the controls in



Text fig. 3 Outlines of heads of a large thyroidless tadpole and of a normal tadpole of a corresponding stage of differentiation.

form, color, vitality or in any other observable character. There is no evidence of any modification in the operated tadpoles until the hind legs begin to grow. These reach a length of 4 to 5.5 mm. and then cease to develop when the tadpoles cease to grow. The fore limbs never break through the skin but are to be found in a most undeveloped condition only after careful dissection. It is thus clear that both the hind and fore limbs can start to develop in the total absence of the thyroid gland but their development ceases at an early stage. In the case of the hind legs, this is quite readily seen. The tail also retains its larval proportions. It is impossible to give any tail measurements of value in this series because as explained, the tails of both thyroidless

and control tadpoles were alike distorted. The tadpole with the straightest tail was killed on February 7. The body measured 43 mm. and the length of the tail was 80 mm. It is thus seen that this tadpole had reached the gigantic length of 123 mm. The shape of the head in front of the eyes undergoes marked change in the thyroidless tadpoles. This can be well seen in the outline drawings made to scale text figure 3. The exact nature of this modification has not yet been worked out but it is a very obvious change. It consists in a lengthening and broadening of this region.

In a number of tadpoles measurements of the alimentary tract were made as given in table 1:

TABLE 1  
*Measurements of alimentary tract*

DATE KILLED	BODY LENGTH	STOMACH LENGTH	SMALL INTESTINE LENGTH	LARGE INTESTINE LENGTH	COMBINED LENGTH STOMACH TO ANUS
Controls					
August 6, no. 3.....	mm. 21.7				mm. 34.5
July 25.....	24 body	8	79	8	95
	59 total				
July 25.....	22 body	8	20	4	32
	29 total				
July 26.....	23	8	15	4	27
Unknown.....	25	10	21	4	35
Unknown.....		8	17	4	29
Thyroidless					
August 6, no. 1.....	15.5				208
August 6, no. 2.....	29				301
October 12.....	30				237
December 6.....	27				190
December 15.....	33	11	174	22	207
February 7.....	43	14	367	45	426
February 14.....	34	7	137	20	164
February 22.....	34	12	244	32	288
March 6.....	40	14	215	22	261
Attempted removal, failure					
August 6, no. 4.....	21.5	13	27	6	46

It will be noted that the thyroidless tadpoles retain the great length of the intestine characteristic of larval life. A comparison of the total length of stomach and intestines of the thyroidless tadpoles shows a large amount of variability that cannot be readily explained. Compare the cases of specimens killed February 7, 14 and 22. A careful study of sections of the thyroid region of these specimens failed to reveal the slightest vestige of a thyroid gland in any case. While the size of the February 7 specimen may partly explain the greater intestinal length this factor does not explain the difference in its length in the February 14 and 22 specimens.

The experiment was made of feeding a thyroid preparation to two of the thyroidless tadpoles. This was administered in the form of dried flour paste in which powdered thyroid had been mixed in the proportion of 1 part of thyroid to 5 of flour. The results are given in table 2:

TABLE 2  
*Length of Limbs*

	BODY LENGTH	LENGTH TIME FED	DATE KILLED	LENGTH HIND LEG	LENGTH FORELEG	LENGTH STOMACH AND INTESTINE
	<i>mm.</i>	<i>days</i>		<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
Control, thyroidless.....	30		October 14	4.5		237
Thyroid fed, thyroidless.....	29	4	October 14	6.4		143
Control, thyroidless.....	30		December 6	4	2.5	190
Thyroid fed, thyroidless.....	31	44	December 6	9.25	5	68

It will thus be seen that tadpoles deprived of the thyroid gland may be retarded in their development for over four months and will then respond to thyroid feeding to at least a certain extent when this is undertaken. This feeding results in a general tendency toward metamorphosis evident in the changed form of the head, the lengthening of the legs and the marked shortening of the alimentary tract. Enough has been accomplished to show that administration of thyroid extracts to a thyroidless tadpole does cause it to resume the development toward metamorphosis that had ceased as a result of the absence of the thyroid gland.



It is my intention to follow out a series of experiments to test the ability of the tadpoles to respond to the stimulus of thyroid feeding at various periods after the cessation of development. This should give us an insight into this very interesting phase of the subject of senescence.

Thyroid removal has a decided effect upon the growth of the brain. This is evident in many features notably as regards the cerebral hemispheres and the optic lobes. A noticeable effect is seen in the development of the cerebellum. The figures speak for themselves. Figure 9 shows the brain of a normal tadpole that has slightly passed the stage at which the thyroidless tadpoles ceased to develop. The total length is 62 mm. and the body length 27 mm. This might well be compared with figure 10, that represents the brain of a tadpole at metamorphosis—body length 24 mm. While the total length of the brain in this case is no greater than in the foregoing, it may be seen that the cerebral hemispheres have undergone a change of form being broadened posteriorly and far more sharply cut off from one another than before. The marked broadening of the optic lobes is especially striking. An increase in the width of the diencephalon is to be seen. With these processes the cerebral hemisphere and the optic lobes have more closely approached one another. Figure 11 shows the brain of a young normal frog of 50 mm. body length killed February 22. This is less than a year old, having undoubtedly metamorphosed the preceding summer. It shows little change in form and proportion over the brain at metamorphosis. The transverse furrow in the cerebral hemispheres is well marked. These have grown backwards over the diencephalon coming almost in contact with the optic lobes. The cerebellum shows a distinct increase in size approximating its final proportions.

Now with this normal development of the brain in mind, it is especially interesting to study the characteristics of the brain of a thyroidless tadpole killed February 7 with a length of 123 mm. and a body length of 43 mm. (fig. 12). One is immediately struck with the immaturity of this brain. This is notably true of the cerebral hemispheres and the optic lobes. These show but

little advance in size and differentiation from the condition found at the cessation of general bodily differentiation as shown in text figure 2, B. The optic lobes have become more erect and rounded; but there is little change elsewhere save in the marked broadening of the diencephalon and myelencephalon. This however is in no sense proportional to the difference in body size. Yet it is in clear contrast to the failure of the cerebral hemispheres and optic lobes to appreciably increase in size.

#### THE GONADS

At the stage when the thyroidless tadpoles cease to undergo bodily differentiation the gonads have already undergone sexual differentiation. Specimens of this stage were not preserved; but studies were made upon controls killed just before and at the time of metamorphosis. These included the specimens given in table 3 whose testes have the dimensions indicated.

It is thus seen that the testes of the thyroidless tadpole killed February 7 are many times as large as those of the control frogs killed at the time of metamorphosis. In structure they are far more advanced. This is especially true of the rete tubules which have joined with the ampullae. The latter have become hollowed out in the thyroidless specimen of February 7 while they are still solid in the normal controls killed at the time of metamorphosis. The most striking advance is in the development of the germ-cells. In the controls at the time of metamorphosis these are in the condition of spermatogonia. There are no spermatocytes visible. In a thyroidless tadpole killed September 14 there are many cells in synapsis and one can find here and there, clusters of spermatids. Careful search, however, did not reveal any spermatozoa. It is unfortunate that there are no controls killed at this time. In the thyroidless tadpole killed September 25, there are some very clear clusters of spermatids. The same conditions are found in the thyroidless specimen of October 10. It is quite certain that no spermatozoa have been formed as yet. The first clear instances of sperm were found in the thyroidless tadpole killed December 15. At this stage, they are still few in number, but in the one killed February 7,

TABLE 3

*Males*

	DATE KILLED	SIZE TADPOLE		SIZE OF TESTES		THICK- NESS	ESTIMATED VOLUME
		Body	Total length	Length	Width		
Controls							
		<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	
MC1	July 25	24	58	R.0.7 L.0.7	0.22 0.21	0.40 0.31	0.0616 0.0456m
MC2	July 25	21	30	R.1.2 L.1.1	0.37 0.38	0.50 0.51	0.2220 0.2132
MC3	August 6	21.5		R.0.8 L.0.7	0.31 0.32	0.40 0.45	0.0992 0.1008
MC4	February 26	56A		R.2.54 L.2.67	0.8658 0.8991	0.57 0.63	1.2424 1.3186
MC5	February 26	56B		R.2.6 L.2.5	0.73 1.03	0.80 0.70	1.5399 1.9591
MC6	February 22	51		R.2.2 L.2.3	0.86 0.63	0.66 0.63	1.2919 1.4297
Thyroidless							
MT1	September 14		97	R.1.2 L.1.1	0.41 0.38	0.48 0.48	0.2522 0.2132
MT2	September 25	36	85	R.1.3 L.1.7	0.32 0.36	0.39 0.44	0.1622 0.2693
MT3	October 10		90	R. L.1.4	 0.26	 0.32	 0.1164
MT4	December 15	33		R.2.5 L.2	0.24 0.31	0.38 0.47	0.2280 0.2914
MT5	February 7	43	123	R.1.7 L.1.9	0.61 0.62	0.90 0.75	0.9333 0.8835
Starved Control							
MS	February 22	27	66	R.0.64 L.0.57	0.27 0.35	0.23 0.26	0.0419 0.0581

very many sperm are present. It is thus clearly demonstrated that spermatogenesis proceeds normally and sexual maturity is attained in thyroidless tadpoles that have retained larval characteristics long after the time for metamorphosis. This is in sharp contrast to the fact that even in the completely metamorphosed frogs killed in July and August, there is no sign of spermatogenesis.

The thyroid region of this thyroidless tadpole killed February 7 was sectioned and it was clearly demonstrated that the thyroid gland was absent. It is interesting to compare the gonads of this specimen with those of two young male frogs killed February 26. These young frogs had transformed the summer before and had lived in the open until captured by the dealers from whom they were purchased. They were secured from a Chicago firm. Although their exact history is not known, it is clear that they are quite as old as the thyroidless tadpoles with which comparison is made. We find that in one of the young frogs of 51 mm. body length, the testes are slightly smaller in transverse section than in the thyroidless tadpole killed over two weeks earlier in the season. In the other frog of 56 mm. body length, the gonads are somewhat larger in transverse section than in the thyroidless tadpole mentioned above, but are roughly proportional to the difference in body length.

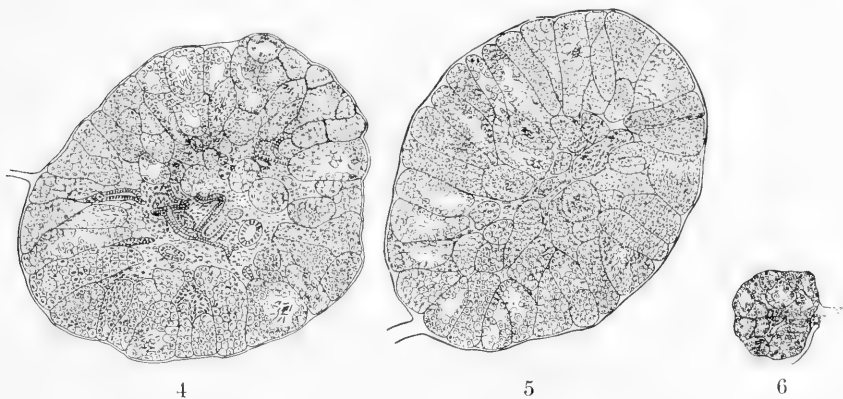
A comparison was made between the volumes of the testes of the older controls and the volume of the testes of the February 7 thyroidless tadpole. It was manifestly impossible to estimate the exact volume, so a rough estimate was made by multiplying together the average width, thickness and length as given in the table, giving a certain numerical basis for comparing the size of these structures. Correction was made for differences in body size by taking an average of the body length of the two classes to be compared, and then multiplying the value found to represent the testis average of the thyroidless tadpoles by a fraction in which the average body length of the controls is the numerator and the average body length of the thyroidless tadpoles the denominator. This does not give a completely accurate basis of comparison, because these body lengths are not comparable, i.e., the body of the tadpole normally shrinks during metamorphosis. The results were as follows:

	<i>mm.</i>
Average of MC <sub>4</sub> , MC <sub>5</sub> , and MC <sub>6</sub> .....	1.4611
Average of the two testes of MT <sub>5</sub> .....	1.1408

Undoubtedly, the thyroidless tadpoles were under a serious handicap as regards nutrition. The malformation of the tails

prevented normal activity in securing food. Taking all of these features into consideration, we are justified in considering the testes of the thyroidless tadpoles to be roughly equivalent to those of the normal frogs that had metamorphosed the summer before.

A tadpole that was starved during the summer, only being fed sufficiently to keep it alive, was fed sparingly from the middle of November up to February 22, when it was killed. It was found to have food in the intestinal tract. The body length was 27 mm. and the total length 66 mm. The legs were 5 mm. long. It had retained the great length of the alimentary tract characteristic of tadpoles, but the gonads were very poorly developed and



Text fig. 4 Transverse section of a testis of a thyroidless tadpole killed February 7.

Text fig. 5 Transverse section of a testis of a young, sexually mature frog 51 mm. body length. Killed February 22.

Text fig. 6 Transverse section of a testis of an unoperated, starved, control tadpole, 27 mm. body length. Killed February 22.

thus in sharp contrast to those of the thyroidless tadpoles. The total volume estimates for the testes of this specimen are 0.0419 mm. and 0.0581 mm., an average of 0.0500 mm. Compare this with the volume estimate of the thyroidless tadpole killed February 7. Here the volume estimates for the testes are 0.9333 mm. and 0.8835 mm. with an average of 0.9084 mm. The latter estimate is roughly 18 times as large as the average testes of the

starvation tadpole which was just as old and was killed 15 days later. Text figures 4, 5, and 6 serve to show the relative degree of development of the testes in a well fed thyroidless specimen, in a normal young frog killed slightly later, and in an otherwise normal tadpole that had been subjected to extreme inanition, respectively. This comparison is quite instructive. It has been shown by Swingle, whose paper appears in this journal, that the germ glands are greatly retarded in their development by poor feeding. If the failure of the thyroidless tadpoles to metamorphose might by some be attributed to inanition, it is possible to refute such an explanation by pointing to the full development of the gonads in the thyroidless specimens, and to the remarkably low development of the testes in this starved specimen which, by the way, had limbs reaching the maximum length of those of the thyroidless tadpoles. When we take into consideration the transformation of the controls kept and fed under identical conditions, the case is seen to be definitely established.

The development of the ovaries and oocytes in the thyroidless tadpoles is equally marked as shown in table 4:

The ovaries show similar conditions, in that they develop in an altogether normal fashion and at a normal rate.

Tadpole FT2 had been fed a thyroid preparation for four days prior to being killed. This has not appreciably modified the gonads, hence it is classed along with the other thyroidless tadpoles. Rough estimates of the dimensions and volume of the ovaries were made. These were by no means so accurate as those made in the case of the testes. It is easy to see why the evident irregularity of the ovaries (text figures 7 and 8) should make this nothing but a crude approximation. Nevertheless, these estimates serve as a very good basis of comparison. In the cases of FC1, FC2, FC3, FC4, FT1, FT2 and FT3, all three dimensions were secured. The length was calculated by counting the number of sections of known (10 mm.) thickness. Width and thickness were measured by taking an average of eye piece micrometer measurements made upon every tenth section in the series.

TABLE 4

*Females*

NUMBER	DATE KILLED	SIZE		DIMENSIONS OF OVARIES				DIMENSIONS OF OOCYTES
		Body	Total	Length	Width	Thickness	Est. volume	
Controls								
FC1	July 25	mm. 22	mm. 63	mm. R. 3.3 L. 2.4	mm. 1.20 1.02	mm. 0.42 0.37	mm. 1.6688 0.9058	0.102 0.133
FC2	July 25	22	29	R. 3.4 L. 1.4	0.80 0.89	0.39 0.51	1.0686 0.6385	0.099 0.122
FC3	August 6	21.7		R. 3.6 L. 3.2	1.46 1.65	0.50 0.42	2.6464 2.2296	0.115 0.136
(Attempted removal) FC4	September 20	19	35	R. 1.7	0.98	0.53	0.8822	0.128 0.157
(Young sexually mature frog) FC5	March 13	48		R. 6.7 L. 7.3	2.04 2.47			0.202 0.222
Thyroidless								
FT1	December 6	35		R. 2.9 L. 5.1	1.42 1.44	0.72 0.64	2.9602 4.7048	0.157 0.184
FT2	December 6	35		R. 3.6 L. 4.1	1.55 1.67	0.74 0.68	4.1882 4.6550	0.147 0.178
FT3	February 14	34	80	R. 4.9 L. 6.2	2.35 2.44	0.90 0.87	10.3667 13.1984	0.197 0.255
FT4	March 6	40	102	R. 7.3 L. 8.4	2.16 2.23			0.206 0.235

In the later stages, as the ovary became very irregular in form, no further attention was paid to the thickness, because such measurements would have very little value.

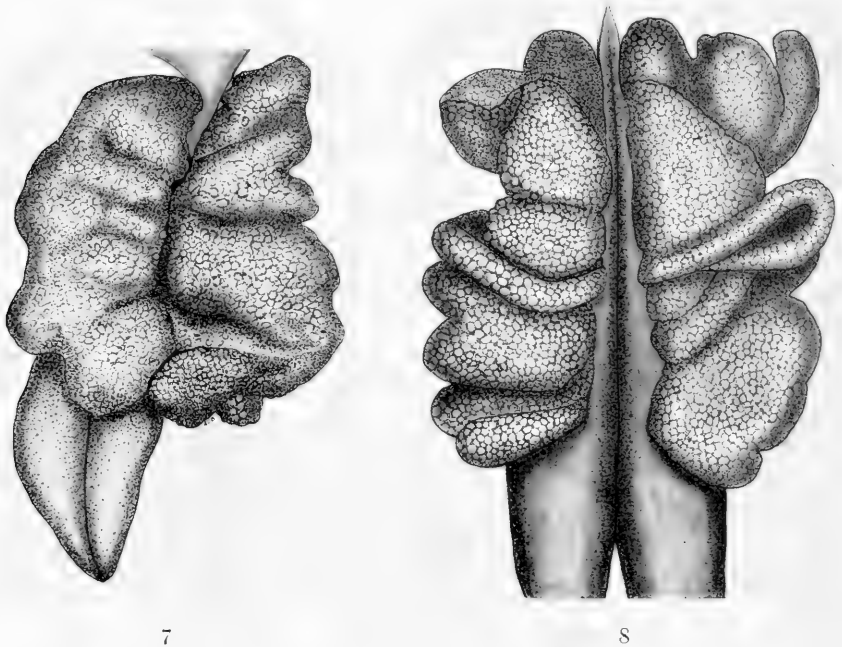
A study of the table measurements shows that the ovaries of the thyroidless tadpoles have continued to increase in size with the passage of time until they have reached a volume well in excess of that of the young normal frogs killed a short time after metamorphosis. The following comparison is instructive.

mm.

Average estimated volume of ovaries of FC1, FC2 and FC3..... 1.5762

Average estimated volume of ovaries of FT1, FT2 and FT3..... 6.5122

Not only is it true that the average volume of the ovaries of the older thyroidless tadpoles is greater than the average of the young metamorphosed controls, but in each individual case this is true. It must be remembered that the thyroidless tadpoles are in an early tadpole stage, in spite of their age and large size, while the controls are completely metamorphosed.



Text fig. 7 Ovary of a thyroidless tadpole with 24 mm. body length. Killed February 14.

Text fig. 8 Ovary of a normal young frog, 48 mm. body length. Killed March 13.

We have seen that there is continued growth and development of the ovary as a whole. There is also marked development of the oocytes. In the thyroidless tadpoles of February 14 and March 6, they are of double the diameter that they exhibit in the recently metamorphosed frogs of July 25. They have become quite easily visible to the naked eye.



Since the female of February 14 is a crucial stage, a series of ten slides through the thyroid region was made and carefully searched for remnants of thyroid tissue. None were found. This demonstrates that this specimen is truly devoid of a thyroid gland.

#### CONCLUSIONS

This work gives results which are in complete harmony with those attained by Gudernatsch, Swingle and others in their experiments in feeding thyroid gland preparations. While Gudernatsch showed that thyroid feeding accelerates development, this work shows that the total absence of the thyroid gland produces complete cessation of somatic differentiation at a certain stage but does not hinder continued growth in size. It is especially interesting in this connection to find that the early organogenesis continues unhindered, up to a certain point. This applies even to the early development of the hind limbs and of the fore limb rudiments. The former undergo the beginnings of ossification, as shown by Terry, while the fore limb rudiments never show the slightest sign of breaking through the skin.

It is evident that the thyroid gland is in no wise essential to the earlier phases of development, but that at a certain definite stage, further development of the soma is dependent upon it. It is impossible to attribute this early normal development to the persistence of secretions given off prior to the operation, because the thyroid anlage is removed at its very inception and the effects do not become evident for from two and a half to three months.

The most remarkable result of this investigation is the demonstration of the sharp difference between its effects upon the gonads and upon the remainder of the body. This had been demonstrated from a different angle by Swingle who, working under my direction, fed thyroid preparations to tadpoles and studied the effects upon the gonads and germ cells. Much to our surprise, they went their normal course unaffected by the great acceleration of body growth. A report on this work was made at the Columbus meeting of the A. A. A. S. in 1915 and Swingle gives a full account of his work in the current issue of this

journal. While he shows that the development of the gonads and germ cells is not accelerated by thyroid feeding, this paper shows that their development is in no wise affected by the absence of the thyroid gland. Swinges shows that the development of the gonads and germ cells is very sensitive to food conditions. In this way, we might explain the incomplete sexual development of cretins. It would seem highly probable that here the thyroid glands are merely the indirect cause, in that they disturb the general bodily growth and metabolism to such an extent as to produce a condition of under nourishment.

It is interesting to speculate upon the bearing of these facts upon Weissmann's hypothesis of the independence of the germ plasm from the soma. Malnutrition naturally affects both, since it regulates the materials that go into the formation of both germ cells and soma. Here with the thyroid gland however, we are dealing with a factor that has to do with the entire process of metamorphosis beyond the stage of early limb development. Just as the thyroid gland is not essential to the earlier phases of embryonic development, so is it likewise not at any time essential to the development of the germ cells. One should not expect the growth of the germ cells to be governed by the thyroid secretion, when the earlier developmental stages of the embryos that arise from them are shown to proceed normally in the absence of the thyroid gland. The whole question of the factors regulating the development of the somatic portions of the gonads is another matter. In *Rana pipiens*, the general features of structure of the gonads are laid down before the period when the soma ceases to differentiate. We know all too little regarding the interesting question of the influence of the germ cells upon the development of the gonads, and it would at present be difficult to say whether the somatic portions of the latter are governed more by the germ cells within them or by the factors that regulate the development of the soma in general.

It will be extremely interesting to analyze further the effects of the thyroid secretion upon the differentiation of organs, upon histogenesis, upon the problem of senescence, and upon regeneration. The present paper shows a most promising method of attack upon the fundamental problems of development.

In these experiments, artificial neoteny has been definitely produced. Sexual maturity was attained in the male and it is altogether probable that it may be reached in the course of time in the female tadpoles. Just as in the classic case of the axolotl, sexual maturity is reached with a persistence of larval form. It is possible that neoteny of the axolotl is due to the failure of the thyroid gland to attain its full development of structure or function.

#### SUMMARY

1. Absence of the thyroid gland in the tadpoles of *Rana pipiens* does not affect the course of early development up to the time when the hind limbs have begun to grow.

2. Further differentiation of the soma then ceases and metamorphosis does not occur. In the present paper, the following features of soma development have been studied with the result that no continuation of differentiation was observed in the following features.

- a. The general body form and limb development.
- b. The alimentary tract in its entirety.
- c. The brain.

Although special studies of other organs were not made, there was no evidence that any of them, except the gonads, showed further differentiation.

3. The anterior portion of the head shows an abnormal form, being lengthened and broadened.

4. Thyroid administration to thyroidless tadpoles brought about a resumption of development even four months after it had ceased.

5. The removal of the thyroid gland has no effect of any kind upon the gonads and germ cells. This is in significant contrast to the effects upon all the other portions of the body.

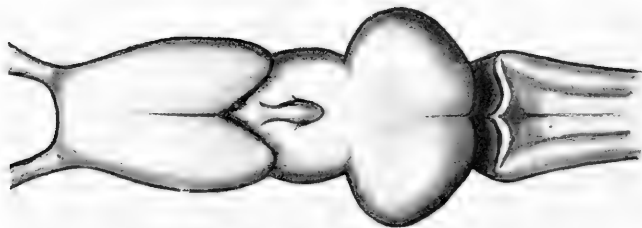
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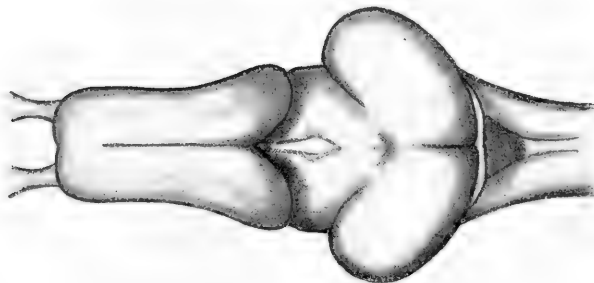
## PLATE 1

### EXPLANATION OF FIGURES

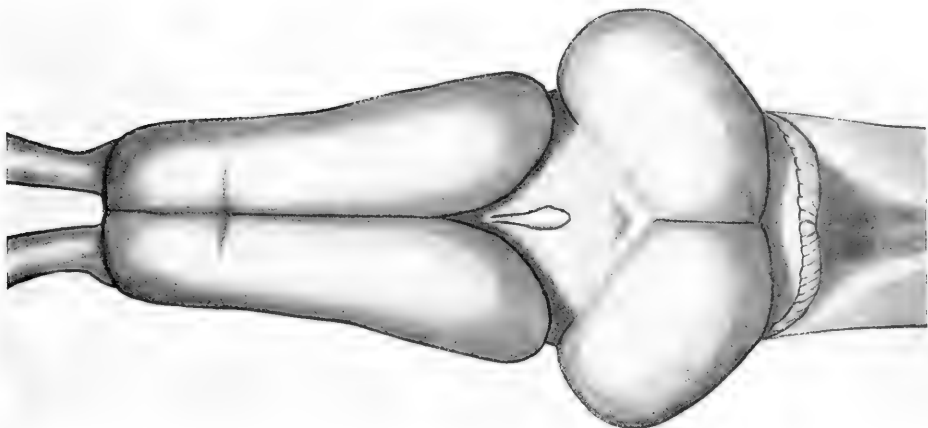
- 9 Brain of a tadpole of 27 mm. body length and 62 mm. total length.
- 10 Brain of recently metamorphosed frog with body length of 24 mm.
- 11 Brain of young frog, 51 mm. body length. Killed February 22.
- 12 Brain of thyroidless tadpole with body length of 43 mm. and total length of 123 mm. Killed February 7.



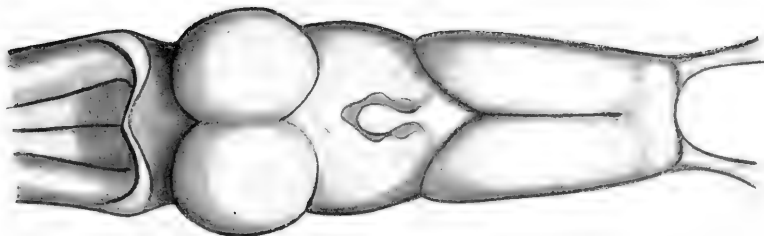
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# THE ACCELERATION OF METAMORPHOSIS IN FROG LARVAE BY THYROID FEEDING, AND THE EFFECTS UPON THE ALIMENTARY TRACT AND SEX GLANDS<sup>1</sup>

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FOURTEEN FIGURES

## INTRODUCTION

Since Brown-Séquard proposed the theory of internal secretion, each successive year has produced an increasing number of researches into the nature and function of the endocrinous glands. One of the striking facts brought out by these experiments, is the physiological interrelation of the various secretory organs. One of the most interesting of these relationships, is the apparent correlation in function of several of the glands of internal secretion, and the reproductive organs. It is fairly well established, for instance, that anomalies and diseases of the hypophysis are in many animals intimately associated with abnormalities of the sex glands and sexual development, and it has been assumed by many investigators that the thyroid gland and its secretory products is also physiologically related to these organs. The functional correlation, however, in the case of the thyroid and gonads is much more obscure than the pituitary-gonad relation, and is, at the present state of our knowledge regarding the problem, open to question.

The results obtained by Gudernatsch, in his experiment upon feeding thyroid glands to tadpoles, by which he was able to produce frogs no larger than flies from immature larvae in a very short time, suggested that frog larvae might be an excellent form for investigating the relation of the thyroid and sex glands.

<sup>1</sup> Reported at the Annual Meeting, 1915, of the American Association for the Advancement of Science, Columbus, Ohio.

The writer thought that by repeating Gudernatsch's thyroid feeding experiment and noting the effect of accelerated metamorphosis on the gonads of young larvae, perhaps some new light might be shed upon the problem of the germ gland-thyroid relationship.

#### LITERATURE

A great many researches dealing with the thyroid gland have appeared in the past few years, but few of them are concerned directly with the relation of the thyroid to the germ glands and cells.

Long ago J. F. Meckel advanced a theory that the thyroid gland is intimately associated physiologically with the female organs of generation. This view is based upon the fact that the thyroid of women is larger than that of men, and that it increases in size during menstruation, deflorescence and pregnancy.

Hofmeister, who investigated the problem of the relation of the thyroid to growth and development, extirpated the glands of young rabbits. Among other pathological changes which resulted, were certain degenerative processes in the reproductive glands; the ovaries, for instance, invariably revealed premature maturity of many of the follicles.

Another investigator, Jeandelize, has shown that in male animals whose thyroid had been removed, there is imperfect development of the testes.

v. Eiselberg found that in sheep whose thyroids had been removed at the age of ten days, there was an inhibition of the growth of the testes. This same author also operated upon goats three weeks after birth, and some months later observed arrested development of the sexual glands.

Lanz observed in his investigations, that after thyroidectomy had been performed upon hens, there resulted imperfect activity of the sexual glands. Such fowl laid very few eggs, and these were abnormally small and covered with very thin shell. This author states that the laying capacity of these thyroidless hens may be increased by feeding with thyroid gland.



Biedl extirpated the thyroids of dogs, three to five weeks of age, and among other changes observed was a noticeable hypoplasia of the sexual glands. This investigator claims that the arrest in development of the sexual organs is one of the typical and invariable results of the absence of the thyroid function in both carnivora and herbivora.

The clinical evidence, derived from cases of athyrosis in man, that the thyroid appears to be related in some way to the sex glands physiologically, is rather striking at first glance. For illustration: One of the most constant symptoms of thyroid insufficiency in young, growing individuals is the infantilism of the sexual organs, in consequence of which puberty is very much retarded or else absent altogether. Furthermore, in myxoedema, a disease due to suppression of the thyroid function, there is frequently disturbance of the sexual functions. Also, in certain other diseases, such as infantilismus myxoedematosus and cretinism, there is usually imperfect development of the sexual organs.

So far as the present writer is aware, the experiments and clinical evidence just presented, represent the extent of present day knowledge concerning the relation of the thyroid to the sex glands. In none of the work quoted here is there any conclusive evidence regarding the direct action of the thyroid secretion upon the gonads; all that is shown is that full sexual development apparently depends upon the presence of the thyroid gland. In view of the physiological relation known to exist between the thyroid and other glands of internal secretion and in turn between the latter and the gonads, it is very probable that the action of the thyroid is indirect and exerts its influence through the intermediation of other glands.

Concerning the effect of thyroid feeding upon frog larvae, there is a growing list of experiments upon these animals since Gudernatsch first published his results. This author fed the larvae of *Rana temporaria* upon thyroid gland and found that the growth of the tadpoles soon ceased, and the tail began to atrophy and metamorphosis began. He was able to produce pigmy frogs, no larger than flies, by thyroid feeding. No report was made as to the condition of the gonads.

Morse fed desiccated thyroid gland to the larvae of *Rana pipiens* and obtained the same results as Gudernatsch. This investigator also obtained positive results by feeding iodised blood albumin, but was unable to produce results with various inorganic iodine compounds. The gonads were not examined.

Lenhart found that the higher the iodine content of the gland fed to the larvae, the more rapid the body metabolism. This observer also found that the thyroid effect is inhibited in tadpoles by cold, or by feeding cracker crumbs.

West fed thyroid tablets to the larvae of *Rana catesbiana* and found that metamorphosis was greatly accelerated.

Barthelmez also fed thyroid to the larvae of various amphibians and obtained positive results, the larvae undergoing metamorphosis more rapidly.

#### MATERIAL AND OBSERVATIONS

The following experiment began April 20, 1915, and continued until June 1, 1916. The larvae of three species of frogs were used in the work: *Rana pipiens*, *Rana catesbiana*, and an undetermined species of the same genus. I shall devote the first portion of this paper to a discussion of the work done in 1915.

April 7, 1915, the eggs of *Rana pipiens* were gathered from a string of shallow pools near the University campus at Lawrence, Kansas, and brought to the laboratory to develop. Most of the eggs were in fairly late segmentation stages and some had developed to the stage of body elongation. When the mouth had formed and the larvae were capable of feeding, one hundred of the tadpoles were selected and divided into two lots of fifty tadpoles each; one lot to serve as controls for the other, which was intended for thyroid feeding purposes. Each group of animals was placed in a large glass fish bowl, 25 inches in circumference by 12 inches deep. Ordinary tap water was used to cover the larvae and was changed daily. Both cultures of tadpoles were kept under uniform conditions of temperature, light and water supply. The larvae of both series of tadpoles averaged the same in length when the experiment began, that is 10 mm. A convenient method of measuring the animals is to lay

them upon moist blotting paper; the moist paper prevents laceration of the tail, and at the same time inhibits the occurrence of the swimming reflex, or lashing movements. April 20 the thyroid feeding began. Various methods of administering the thyroid extract were tried. The first method was to place the powdered thyroid in the water and allow the larvae to eat it. This manner of administering the extract soon proved unsatisfactory because of the scum which forms over the surface of the water, shutting out the oxygen and causing great mortality among the larvae. Another method which proved eminently satisfactory and was employed throughout the experiment, was to mix the powdered thyroid obtained from Armour and Company with wheat flour, in the proportion of 3 grams of thyroid to 10 grams of flour, with the addition of sufficient water to make a thick paste. The paste was then spread thinly over glass plates and allowed to dry at room temperature. Pieces of the dried paste, the size of one's little finger nail, were finely crumbled and fed to the larvae. The control animals were fed bits of fresh beef liver, in order to equalize the amount of protein material fed the thyroid animals, and large quantities of algae.

The thyroid-fed animals ceased to grow after the administration of the thyroid-flour-paste, and five days from the date of the first feeding, differed considerably in appearance from their controls. The bodies of these thyroid fed larvae appeared slender and the head more elongated than that of the control; all of the larvae revealed indications of tail involution. Eight days after the administration of thyroid, limb buds were observed when the animals were examined with the hand lens. The pigmentation had increased somewhat in the thyroid-fed tadpoles as they appeared darker than the algae-fed larvae. The elongated appearance of the head, previously noted, had increased; the body had become emaciated and pronounced atrophy of the tail was observed. The limb buds were now plainly visible without the aid of the hand lens. When contrasted with the thyroid-fed larvae, the controls revealed none of the changes enumerated, and had increased in size somewhat, during the eight day interval of the experiment.

Twelve days after the first thyroid extract was fed, the larvae of both thyroid-fed and control cultures were measured. The average length of the experimental larvae was 7 mm., of the controls 16 mm. The tadpoles fed with thyroid had diminished in length 3 mm.; the normal larvae had increased in length 6 mm. The differences between the two series of animals were very great. The tails of the thyroid-fed larvae had shriveled greatly, and in three larvae had been almost completely resorbed. Many of the larvae experienced great difficulty in swimming because of their rudimentary tails. The hind limbs were very noticeable, though as yet they had not completely differentiated into their two primary divisions. Other indications of metamorphosis were apparent: the sucker-like tadpole mouth had begun to assume the shape typical of that of the adult frog; the elongated appearance of the head was found, upon examination of the viscera, to be due to a remarkable atrophy and resorption of the long coiled gut.

The intestines of thirty of these thyroid-fed larvae and thirty of the normal animals were measured; the entire length of the gut was included, from the oesophagus to the anus. In the thyroid-fed animals the average length of the alimentary tract was 20.5 mm., whereas in the controls the average length of the gut in thirty larvae was 50.5 mm. The appearance of the gut of the thyroid-fed larvae differed remarkably from that of the controls in that it was very like the alimentary tract of the adult form. The gut no longer presented the appearance of a long coiled tube of about equal diameter throughout its entire length; instead, the typical frog stomach and large and small intestine had differentiated.

Besides these changes in the viscera, there appeared to be certain marked growth changes in the bones of the head. These changes, however, were not investigated.

The mortality among the thyroid-fed larvae became very great after the twelfth day of thyroid feeding. Each morning four or five larvae were found dead in the containers.

May 5, or just fifteen days from the date the experiment began,

the larvae of both thyroid-fed and control cultures were killed, and preserved for microscopic examination of the germ glands.

The fixing fluids used were potassium bichromate-acetic, and Flemming's solution; both gave excellent results. The sections were cut a thickness of  $7.5\mu$  and stained with Heidenhain's iron hematoxylin. A counterstain of congo red was also employed.

Microscopic examination of the gonads of both thyroid-fed and control larvae revealed nothing of importance. The glands of the various tadpoles of each series varied somewhat in size; there appeared to be no constant size of the glands among the animals as a whole, or of each group. Figure 1, represents a cross section through the gonad of a thyroid-fed animal. When this diagram is compared with that of figure 2 (transverse section through gonad of a control) it is obvious that the marked somatic changes brought on by the administration of thyroid extract do not extend to the gonads and germ cells. The gonads of the animals of both series are in practically identical stages of development. The diagrams shown in figure 1 and 2 were taken from representative portions of each gonad. In some of the thyroid-fed animals the glands contained more germ cells than are figured in the cross section of the animals chosen, but this was also true of some of the controls.

April 28, another series of tadpoles was selected for thyroid-feeding. These larvae were somewhat larger than the animals used in the work just described, averaging 15 mm. in length at the beginning of the experiment. Eighty animals were used, forty for thyroid-feeding, forty for controls. The method of procedure was the same as that reported for series 1.

May 8, the peculiar body shape characteristic of thyroid fed tadpoles became noticeable; also the limb buds of the hind legs appeared. The controls showed none of these changes. By May 12, all of the larvae of the thyroid-fed culture were well along in metamorphosis. Such rapid somatic changes, and consequent accelerated metabolism, was accompanied by great mortality among the animals. The following table gives the measurements of the larvae, and also the amount the spiral gut

shortened in the fourteen day interval of the experiment in the thyroid-fed group as contrasted with their controls:

*Series killed May 12*

LENGTH OF THYROID-FED LARVAE	LENGTH OF CONTROLS	LENGTH OF GUT OF THYROID-FED	LENGTH OF GUT OF CONTROLS
<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
15.0	21.0	24.0	78.0
12.5	30.0	20.0	80.0
13.0	25.0	26.0	90.0
14.5	23.0	23.0	70.0
15.0	22.0	21.0	75.0
14.0	24.0	20.0	65.0
13.5	25.0	22.0	67.0
12.5	21.0	25.0	74.0
12.0	23.0	23.0	78.0
13.0	24.0	21.0	67.0
14.0	25.0	24.0	65.0
15.0	22.0	22.0	70.0
12.0	21.0	25.0	71.0
11.5	20.0	27.0	73.0
11.0	23.0	25.0	75.0
Average 13.23	23.2	23.2	73.2

Microscopic examination of the gonads and germ cells revealed no observable differences between those of the thyroid-fed and control larvae. This is rather striking, considering the very great bodily changes rapidly taking place in those animals given the thyroid diet. Even in the case of animals in which metamorphosis had reached an advanced stage, there were no changes in the condition of the gonads and germ cells observed, differing from their controls. Body metamorphosis, in other words, had not affected the germ glands or germ cells in any way.

On May 8, 1915, eighty larvae, averaging 25 mm. in length were divided into two lots of forty each; one for thyroid feeding, the other to serve as controls. The feeding began the same day.

May 16, the typical changes of hyperthyroidism made their appearance in all of the thyroid-fed larvae. The feeding was

continued until May 25th. Upon this date, all of the thyroid-fed larvae remaining alive were killed and preserved for sectioning. They averaged in length 20.5 mm. The tails of most of them were hardly more than a mere stub. The fore and hind limbs were well developed; in fact metamorphosis was almost complete. The young frogs, however, were very small.

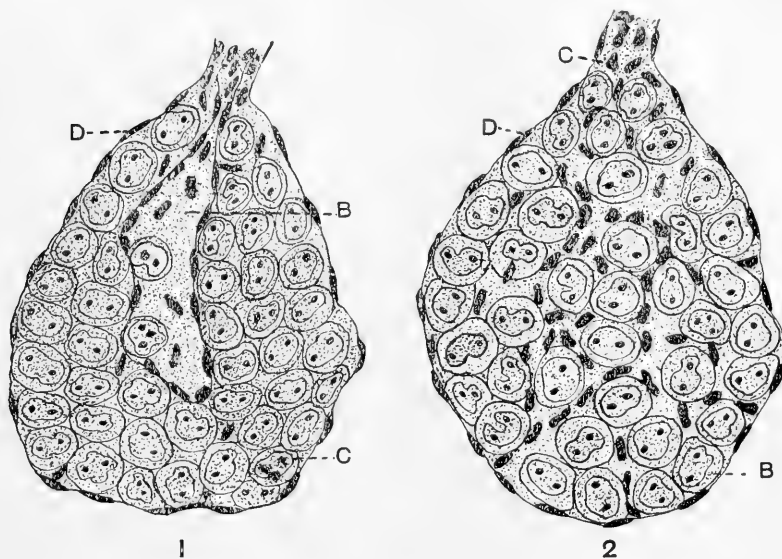


Fig. 1 Representative transverse section through the gonad of a thyroid-fed larvae in advanced stage of metamorphosis. Fed thyroid fifteen days. *D*, follicle cell; *B*, secondary genital cavity; *C*, germ cell.  $\times 800$ .

Fig. 2 Transverse section through the gonad of the control for animal described in figure 1. *D*, follicle cell; *B*, germ cell; *C*, mesenchyme cell.  $\times 800$ .

The control animals for this series showed none of these changes but instead, had doubled their length, averaging 41.5 mm. There were no indications of metamorphosis other than the tiny limb buds of the posterior extremities. These buds usually make their appearance when the animal is about 25 mm. in length. The limbs, however, had not differentiated into their two primary divisions, nor had the toes appeared (fig. 3).

Microscopic examination of the gonads and germ cells of both control and thyroid-fed larvae revealed no observable

differences either in regard to the size or development of the glands or germ cells (figs. 4 and 5). Measurements of the spiral gut of the two groups of larvae, as was noted for earlier series examined, revealed remarkable atrophy of the alimentary tract of the thyroid-fed larvae. In these animals, the typical frog gut had replaced the simple, tube-like digestive tract of the larval form.

No further work was done along this line, until the late summer and early Fall of 1915, when feeding experiments were carried



3

Fig. 3 Photograph of thyroid fed and control larvae. The large tadpoles are the controls. The black color of the experimental animals is partly due to their having been fixed in Flemming's solution.

out with *Rana catesbiana* and one group of larvae, the species of which has not been determined. I shall discuss the experiments upon the unidentified larvae first, because their growth rate more nearly resembled that of *Rana pipiens*.

November 17, 1915, a new series of very young larvae were started upon the thyroid diet. These animals, eighty in number, were found in a shallow pool near the University, and, judging from the size of the pool which was hardly more than 2 feet wide, and of the larvae, which varied but a few millimeters in length, appeared to be from the same batch of eggs. The



average length of the tadpoles was 17 mm. Fifty of these larvae were selected for thyroid feeding purposes, and divided into lots of twenty-five each, one group to serve for controls. Both groups were placed under identical environmental conditions. The first administration of thyroid was on November 17.

All of the body changes, characteristic of thyroid-fed larvae, soon appeared, and by November 25th, eight days after the first

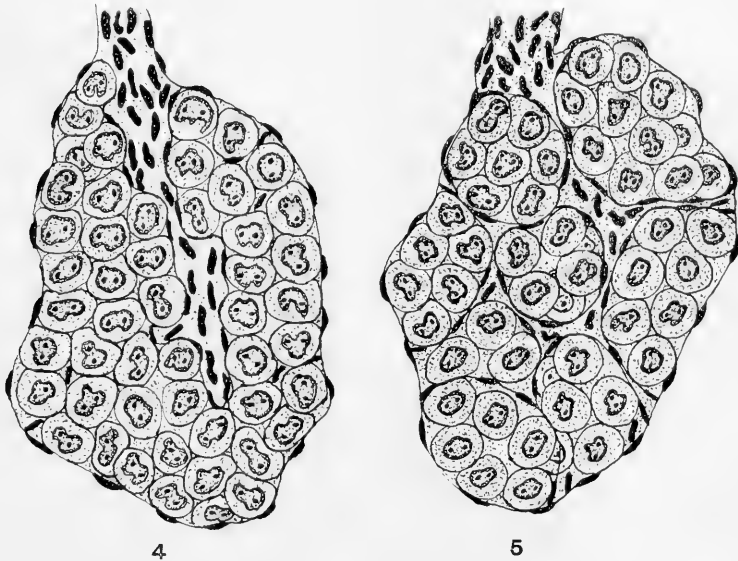


Fig. 4 Representative transverse section through the gonad of thyroid-fed animal in advanced stage of metamorphosis.  $\times 800$ .

Fig. 5 Transverse section through the gonad of control for animal shown in figure 4.  $\times 800$ .

administration of the thyroid, limb buds of the posterior extremities were clearly discernable. When measured, the thyroid-fed larvae still retained their length of 17 mm.; thus indicating that growth had ceased when the thyroid feeding began. Other features characteristic of hyperthyroidism observed at this time were: protrusion of the eyes, elongated appearance of the head, atrophy of the tail, and increased pigmentation.

The controls of this group of larvae, averaged 20.5 mm. in length, showing an increase in length of 3.5 mm. during the

eight days of the experiment. None of the controls revealed any indications of limb development, nor any other of the structural changes exhibited by the thyroid fed larvae.

November 20, marked atrophy and degenerative changes of the tail became evident, the organ was so shriveled as to be practically functionless. The sucker-like tadpole mouth had given place to the mouth characteristic of the adult. About this stage, two small prominences were observed under the skin of the pectoral region. These proved upon dissection to be the fore limbs.

From November 30, on, the mortality among the thyroid-fed larvae greatly increased, until by December 7th, only seven larvae remained alive; six of these were killed and preserved for microscopic examination of the gonads.

The one remaining thyroid-fed tadpole was kept alive by careful dieting, until December 16. During this time no further metamorphic changes other than those recorded were observed. The limb buds grew somewhat in length and differentiated into their primary divisions. The animal died a few days later.

The control animals for the thyroid-fed tadpoles just described had, by December 7, increased in size considerably. The average length at this time was 28 mm. Two larvae measured 33 and 34 mm. respectively. All of the control animals possessed tiny limb buds, though not nearly so large as those of the thyroid-fed group. The fore limbs had not begun to form at this time.

Microscopic examination of the gonads revealed little difference in size or development between the glands and germ cells of the thyroid-fed and control larvae.

It was impossible to obtain a record of the normal rate of growth and development of the gonads in the short space of time the experiments continued, which was twenty days for the oldest larvae. It matters little, however, whether the normal rate is slow or rapid, thyroid feeding did not accelerate growth or development of the germ glands and cells of the experimental animals over that of the controls. As was the case with the

feeding experiments with *Rana pipiens*, thyroid feeding greatly accelerated metamorphic changes, it had no effect upon the gonads and germ cells. The manner in which the body changes occurred in the thyroid-fed larvae of the unknown species was identical with those observed for *Rana pipiens*, and seems to be characteristic reaction of frog larvae to thyroid feeding.

August 4, 1915, a number of larvae of the common bull frog, *Rana catesbiana*, were brought to the laboratory. The animals averaged 35 mm. in length. None of the animals revealed any indications of limb buds. Thirty-five of the larvae were selected for thyroid feeding, with a similar number of controls. The method of administering the extract was the same as that described for the previous experiments, the larvae of both experimental and control groups were kept in the sunlight as much as possible.

August 14, ten days from the beginning of the experiment, metamorphic changes characteristic of hyperthyroidism appeared. The bodies of the larvae were somewhat emaciated, the heads elongated and all of the animals had limb buds.

By August 18, these metamorphic changes had become very pronounced. The tadpole mouth was transforming into that characteristic of frogs; the tails of the larvae were considerably atrophied; and the fore limb of several animals had appeared. The animals died a few days later.

None of the changes just described were observed in the animals of the control group.

When one considers that it requires two and sometimes three seasons for a *Rana catesbiana* tadpole 35 mm. long to develop to the period of complete metamorphosis, the extreme rapidity with which metamorphic changes were brought on by the thyroid stimulus is startling. The intense sunlight undoubtedly acted in accelerating the effect of the thyroid, for I have never been able to induce metamorphic changes so rapidly since.

When the germ glands of the thyroid-fed and control larvae were examined macroscopically, no differences worth recording were observed. The glands of the animals of both groups varied from one another somewhat in size. Taking the groups as a

whole, however, the gonads of the two series of animals appeared to be in the same stages of development.

No microscopic examination of the germ cells was made.

September 23, a second series of *Rana catesbiana* larvae was started upon the thyroid diet—appropriately controlled. The method of administering the extract was the same as that already described. The larvae averaged in length, September 23, 75 mm., most of the tadpoles had small hind limb buds, though as yet they had not developed into their two primary divisions nor had the toe points differentiated.

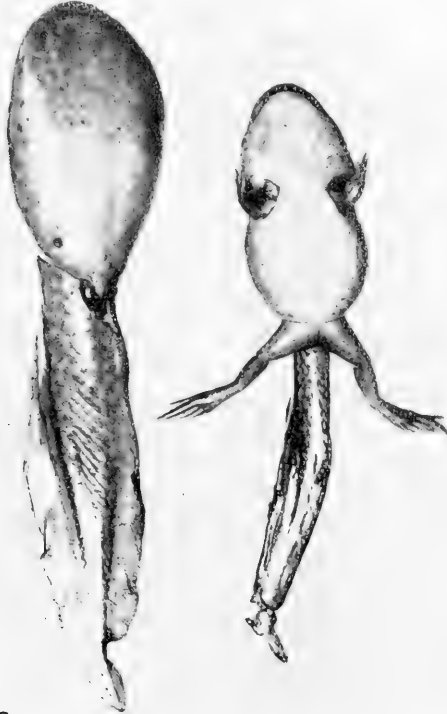
October 10, the thyroid-fed tadpoles exhibited all the symptoms of hyperthyroidism characteristic of these animals. The tails of the larvae were somewhat atrophied and frayed along the edges; the heads appeared elongated; the bodies were darkly pigmented and greatly emaciated. All of the larvae had large, well developed hind limbs, averaging in length 11.5 mm. In some of the animals, the fore legs had broken through the skin and though very small, they seemed well formed. The tadpole mouth showed signs of breaking down to form the mouth characteristic of the adult. The urostyle was plainly visible on all of the thyroid-fed animals. As is obvious from the description, all of the experimental animals were well along in metamorphosis.

October 16, the developmental stages enumerated for the animals of the thyroid-fed group were very pronounced. The tails of all the larvae were undergoing resorption. In two of the animals, the tails were mere stubs, the animals having completed metamorphosis. The mortality among the animals became so great at this time that it was considered advisable to kill all but two of the thyroid-fed tadpoles, together with a similar number of the controls, and preserve them for microscopic examination of the gonads.

When killed, most of the animals had nearly completed metamorphosis, whereas the control animals had increased in size 7.5 mm. during the interval the experiment continued. There were no indications of metamorphosis in the algae-fed controls. In figures 6 and 7 are shown the thyroid-fed and control animals of this group. The developmental differences between thyroid-

fed and algae-fed animals are very marked. The experimental animals are far ahead of the control tadpoles in somatic development.

The alimentary tracts of both control and thyroid-fed animals



6

Fig. 6 Photograph of thyroid-fed and control *Rana catesbiana* larvae. Fed thyroid 23 days.

were measured. The average length of the tract from oesophagus to cloaca in the experimental larvae was 100 mm. In all the thyroid-fed animals, the shape of the gut was very similar to that of the adult. Figure 8 shows that the gut had differentiated into the divisions characteristic of the frog, and had a well developed stomach, a small and a large intestine.

In the control animals, the average length of the spiral gut

was 346 mm. The alimentary tract was simply a long coiled tube, with no differentiation into stomach, small and large intestine. When one pauses to consider that in the short space of two weeks or so the gut of the thyroid-fed larvae actually decreased 246 mm. in length, and differentiated from a long coiled tube into a stomach, a small intestine and a large intestine, one is astonished indeed (figs. 8 and 9).

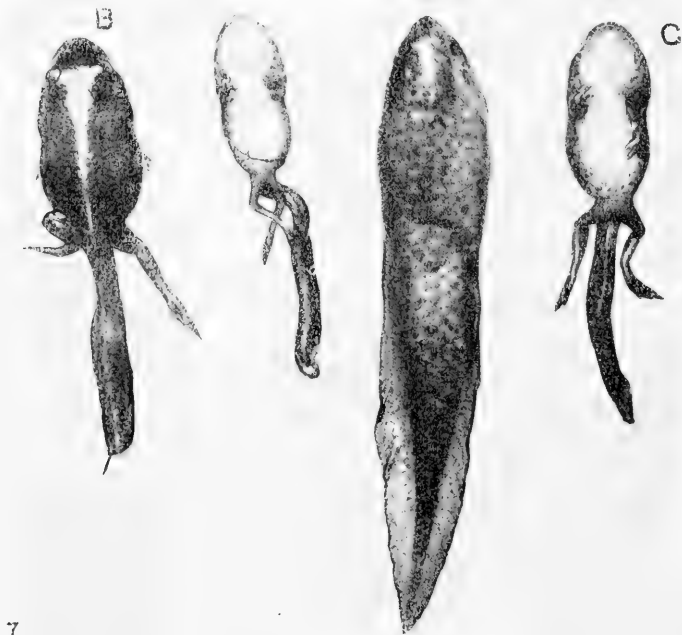


Fig. 7 Photograph of same series of animals as shown in figure 6.

Macroscopic examination of the gonads of both experimental and control larvae, revealed considerable variation in regard to the size of the glands among the animals of each series. Only animals of the same sex were compared. The germ glands of the thyroid-fed larvae were slightly smaller than those of the control animals. In female tadpoles, a comparison of the ovaries of the normal and thyroid-fed animals showed fewer convolutions in the glands of the thyroid series. The number and depth of

the convolutions in the ovaries of frog larvae appear to be an indication of their development. In figure 10 is shown the gonads of a thyroid-fed larva, which was well along in metamorphosis when killed. These gonads were taken from the animal shown in figure 6. When they are compared with the gonads shown in figure 11 taken from the control animal shown in figure 6

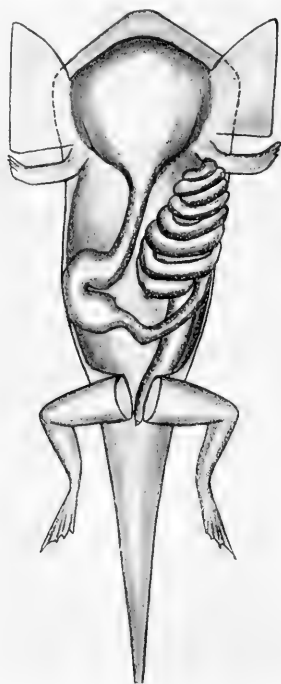


Fig. 8 Alimentary tract of thyroid-fed larva marked *C* in figure 7. Length of gut 100 mm.

it is obvious that the acceleration of body development has had no effect upon the gonads. That the germ cells were unaffected is shown by a comparison of figures 12 and 13. These figures are diagrams of representative transverse sections through the gonads figured in 10 and 11. The germ cells of both thyroid-fed and control larvae are in identical stages of development. In

both animals the center of the glands are filled with growing oocytes, while at the periphery are cells in synaptene stages.

Another feeding experiment of a somewhat different nature from the preceding, was tried upon *Rana catesbiana* larvae. The larvae, measuring 75 mm. in length, with hind limb buds 3



Fig. 9 Alimentary tract of control for animal shown in figure 8. Length of gut 346 mm.

mm. long and normal in every way, were starved from November 1, 1915 until April 8, 1916—a period of over five months. Nothing was fed these animals during this time. At the end of the period of inanition, the tadpoles averaged 72.5 mm. in length. The reduction in length was due to the resorption of the tail. The larvae were greatly emaciated; the intestines had shriveled; the skin over the back appeared corrugated and wrinkled, as though the musculature beneath had been absorbed. The



limb buds had not increased in size; the tails of the larvae had dwindled to half their original width of November 1. The larvae were very sluggish and swam about only when violently disturbed. April 8, 1916, five of these starved tadpoles were

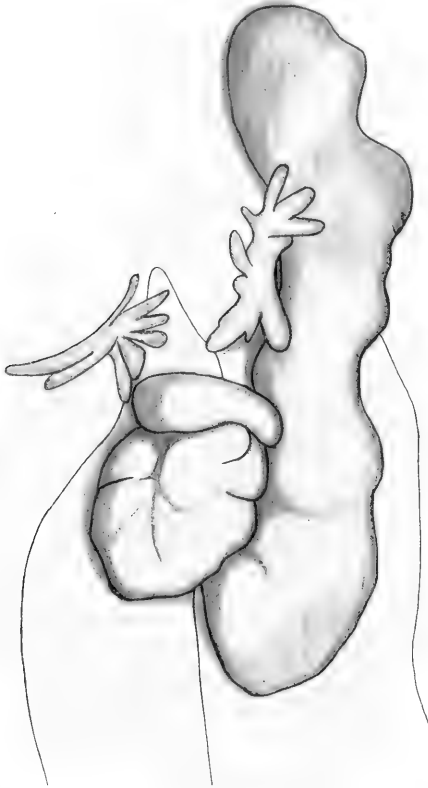


Fig. 10 Gonad of thyroid-fed larva. From animal shown in figure 9.

started upon the thyroid-flour-paste diet; the remaining five animals were fed corresponding amounts of flour paste without the thyroid extract, and in addition, finely shredded pieces of beef liver.

At the end of the ten days, the thyroid-fed animals showed indications of the typical thyroid reaction. Twenty days from

the date of feeding, the tails of the thyroid-fed tadpoles were much atrophied and the hind limbs had grown considerably. Twenty-six days from the date of the first administration of thyroid, the fore limbs had broken through the skin; the tadpole

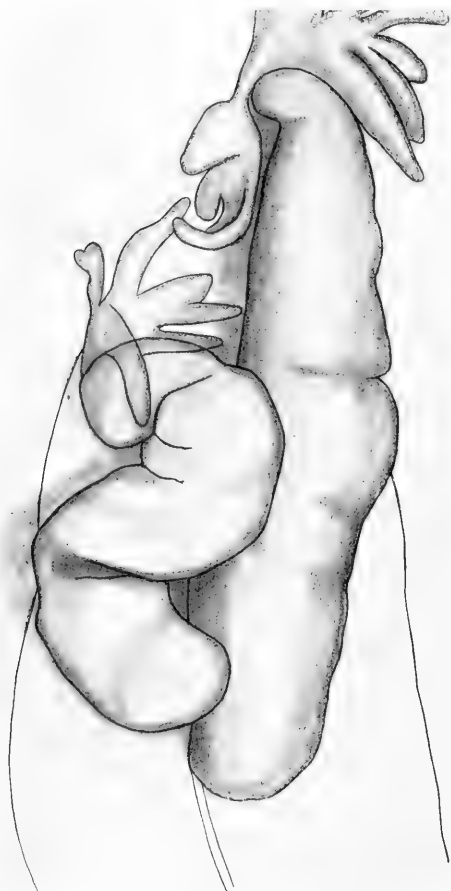


Fig. 11 Gonad of control for thyroid-fed larvae shown in figure 9.

mouth had given place to that of the frog. The tails of these animals had suffered considerable atrophy. By May 4 all of the thyroid-fed animals had died. Figure 14 is a photograph of this series of starved larvae and their controls. The controls for

this series showed little or no change during the period of the experiment, and when measured May 1, had increased their length about 3 mm.

It seems odd that after four months of total starvation, during which growth and development were inhibited, the feeding of thyroid extract should stimulate these starved animals to undergo almost complete metamorphosis.

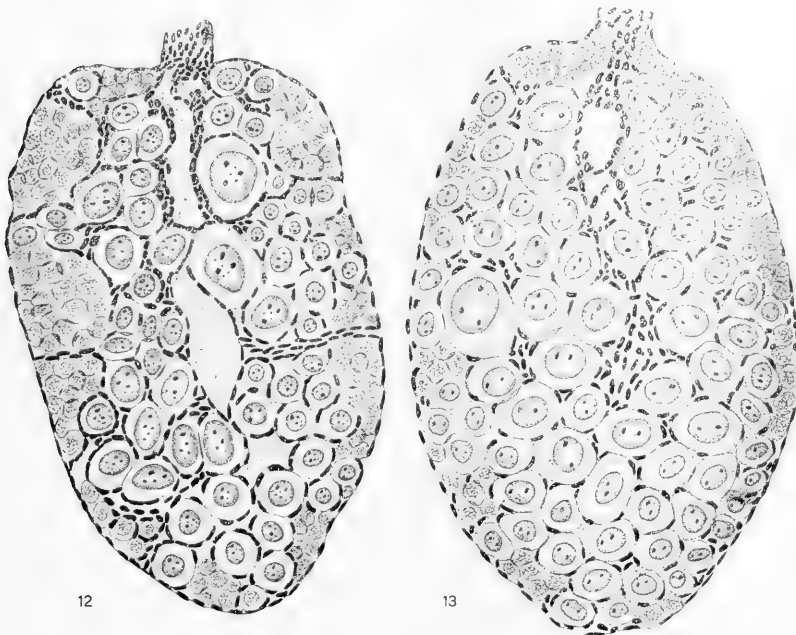


Fig. 12 Representative transverse section through the gonad of thyroid-fed larvae which had almost completed metamorphosis.  $\times 800$ .

Fig. 13 Representative transverse section through gonad of control larva for animal shown in figure 12.  $\times 800$ .

### CONCLUSION

The results obtained in the present experiment appear to indicate that the thyroid gland has no intimate physiological relation to the gonads and germ cells in the amphibia. By feeding thyroid extract to frog larvae, great bodily changes are brought about within a very few days; entire organs and systems

of organs are transformed with startling rapidity from the larval condition to that characteristic of the adult, and yet in the midst of such somatic transformations, the gonads and germ cells remain unaffected.

In a state of nature it requires two seasons and sometimes three, for the larvae of *Rana catesbiana* to reach the adult condition. It is possible by judicious thyroid feeding, to bring



Fig. 14 Photograph of starved thyroid-fed larvae and their controls. The animals in the center are the thyroid-fed tadpoles.

about almost complete metamorphosis within a period of three weeks in the immature larvae of this species of frog. The animals have all the body characteristics of the metamorphosed frog, yet the germ cells and germ glands are those of young larvae.

In conclusion I wish to acknowledge my indebtedness to Dr. B. M. Allen of the University of Kansas for suggesting this problem and for the interest he has shown in the work.

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## THE EFFECT OF INANITION UPON THE DEVELOPMENT OF THE GERM GLANDS AND GERM CELLS OF FROG LARVAE<sup>1</sup>

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FOURTEEN TEXT FIGURES

The following experiment was undertaken with the object of determining whether prolonged starvation of larval frogs has any effect upon the development of the gonads and germ cells of these animals.

Frog larvae are excellent forms with which to work in any study of inanition; the tadpoles are easily handled, require little attention, and the period of larval life offers good opportunities for one to gauge the influence of starvation upon any organ or organ system.

### LITERATURE

There is a large amount of literature dealing with the general subject of inanition, but so far as the present writer is aware, there is but one brief mention of the effects of starvation upon the germ cells and the gonads of larval frogs. This reference is a brief record by Nussbaum of some experiments he performed in the late seventies. In view of the relation between his work and the present experiment, I shall quote him verbatim:

Die sogleich zu beschreibenden Veränderungen der primären Anlage sind durchaus typische, indem sie in derselben Reihenfolge an einer sehr grossen Zahl von Exemplären in zwei aufeinander folgenden Jahren beobachtet wurden. Allgemein gültige Angaben über zeitliches Auftreten, sowie präzise Bestimmungen der Beziehungen zum Erscheinen gewisser anderer Anlagen, beispielsweise der Beine, lassen sich

<sup>1</sup> Reported at the annual meeting 1915, of the American Association for the Advancement of Science, Columbus, Ohio.

jedoch nicht machen. Man muss von Tag zu Tag aus einer reichen Brut mehrere Exemplare untersuchen und wird alsdann die Entwicklung der Organe in der Folge sich abspielen sehen, wie sie unten wird geschildert werden. Zum Beweise wie trüglich äussere Merkmale, führe ich Folgendes an. Im Winter 1877/78 zog ich zwei Bruten getrennt, von denen die eine gut, die andere nur kümmerlich sich nähren konnte. Untersuchte ich nun die gut genährten, deren Hinterbeine schon die Anlage der Zehen zeigten, so fand sich in den Geschlechtsdrüsen dasselbe Stadium wie in den schlecht genährten, obwohl die letzteren nichts weiter als jene weisslichen Höckerchen zur Seite des Afters die erst nur mit der Loupe sichtbare Anlage der Hinterbeine aufwiesen. Man kann also nicht mit Sicherheit bestimmen, welcher Zustand der Geschlechtsdrüsen bei diesem oder jenem Entwicklungsgrade der Larve wird gefunden werden; man ist dagegen wohl im Stande anzugeben, welche Veränderung einem bestimmten Zustande vorausgeht oder folgen wird. Zugleich zeigt aber auch der obige Versuch, welche grosse Rolle in der thierischen Oekonomie die Geschlechtsorgane spielen: das Individuum verkümmert wegen mangelnder Ernährung; die Geschlechtsdrüsen entwickeln sich weiter.

More recently, Nussbaum ('06) has published an account of some inanition experiments he performed upon adult male frogs. He found that starvation, though it reduced the gross size of the testes of his experimental animals to one-third of their original size, did not completely suppress the tendency of the germ cells to multiply.

As was stated before, a search through the literature dealing with inanition, with the exception of Nussbaum's experiments, has failed to reveal any further work done along this particular line.

One investigator, Mead ('00) working with starfish, obtained great size differences between his fed and unfed animals. He concluded from the results obtained, that the difference in growth of different individuals is due to variations in the amount of food supply. This author also finds that sexual maturity in the starfish is correlated with the attainment of a certain size.

Jackson ('15) finds that in young rats held at constant body weight by underfeeding, there is an increase in the weight of the testes, but that in adult rats, the testes apparently lose weight in about the same proportion as the entire body.

In case of the ovaries of young rats three to ten weeks of age,



held at constant body weight, there is a considerable decrease in weight (27 per cent in absolute weight). The author remarks, however, that the ovary is an extremely variable organ, and difficult to dissect out with accuracy.

Another investigator, Hatai ('15), used a chemically defective (lipoid-free) diet upon albino rats, and found a marked atrophy of the testes in those animals whose growth had been retarded by the lipoid-free ration.

Falck ('54) experimented with dogs, and found that the relative (percentage) weight of the testes of these animals is unaffected by inanition.

Voit, working with cats ('66), found that there is a relative decrease in the weight of the testes of these animals when they are subjected to inanition.

Grandis observed in fasting pigeons, that spermatogenesis ceased after the beginning of the inanition experiment. He found that the sperm cells already formed soon died and were resorbed, as was also the greater portion of the seminiferous tubules. Some cells in the walls of the tubes persisted however and after completion of the fast and renewed feeding, these remaining cells gave rise to new sexual elements. This author expresses the opinion that testis cells assume the character of embryonic cells during periods of inanition.

Semonowitch starved a series of rabbits and guinea pigs and found, upon examination of the testes, a parenchymatous modification (slight swelling and granular degeneration) which was associated with fatty degeneration, vacuolization of the cells and chromatolysis. These degenerative changes, however, appeared irregularly in the starved animals. Functional spermatozoa were found in the seminiferous tubules. All of the degenerative changes vanish, the author states, upon renewed feeding.

Cordés found that in conditions of chronic illness in man, which produce cachexia, there is an increase of interstitial tissue in the testes, and thickening of the walls of the seminiferous tubules. In twenty-one cases, examined by him, without cachexia, spermatogenesis had ceased. In very emaciated cases he found cessation of spermatogenesis.

Contrary to the findings of most of the investigators, recorded above, Traina maintains that the changes which the testes of starved animals suffer are by no means marked. Even where there is a loss of weight as high as 20 to 25 per cent, spermatogenesis is maintained in adult animals. This author states that only when the loss of weight becomes as high as 30 to 35 per cent does spermatogenesis cease, but that even then one finds division figures in the spermatogonia and spermatocytes.

Von Hansemann observed that in hibernating marmots, spermatogenesis ceases, and that the interstitial cells almost disappear. In certain non-hibernating animals, on the other hand, the interstitial cells of the testes are very abundant.

Morpurgo ('88) found that in animals subjected to inanition, cell multiplication persists longer in the sex glands than in any other organ or tissue.

Schultz ('04) has shown that the male germ cells of *Planaria lactea* are more resistant to the effects of starvation than are other body cells.

In many of the investigations just quoted, no results of microscopic examination are reported, and in others, where such examinations were made, the results conflict. This rather extensive review of the literature is thought justifiable in view of the disparity of the results obtained by various investigators.

#### MATERIAL AND OBSERVATIONS

Throughout the present experiment, the larvae of *Rana pipiens* were used. They were reared from the egg in the laboratory, in order that the exact age of each tadpole could be determined.

Two groups of eggs were started the same day, the larvae developing from one culture were destined to serve as future controls for the other group. Both series of eggs were kept under uniform conditions of light, temperature and water supply, throughout the experiment. The eggs developed rapidly, and when the young larvae had escaped from their gelatinous capsules and developed to the free feeding stage (which occurred about April 10) the animals of the culture intended for controls

were each day fed algae, the tadpoles of the other groups received no food of any kind.

The growth of both control and starved larvae ran parallel in regard to size, for about six days, i.e., until April 16.<sup>2</sup> The increase in size of the unfed tadpoles was probably due to the consumption of the surplus yolk material held in reserve within the body cells. From April 16 on, however, the algae-fed tadpoles increased rapidly in size, as compared to the starved animals, and by April 27 were twice the size of the unfed larvae.

On May 6, ten tadpoles of both the control and starved cultures were killed and preserved for microscopic examination of the gonads, by fixation in Flemming's solution.

The control animals at this time measured 32 mm. in length, whereas the starved tadpoles averaged but 12 mm. thus showing a length difference of 20 mm. between the animals of the two series. The controls revealed indications of limb development; the limb buds of the hind legs were clearly discernible as small, fleshy projections, just anterior and lateral to the anal opening. A careful examination with the microscope failed to show any indications of limb development among the starved larvae.

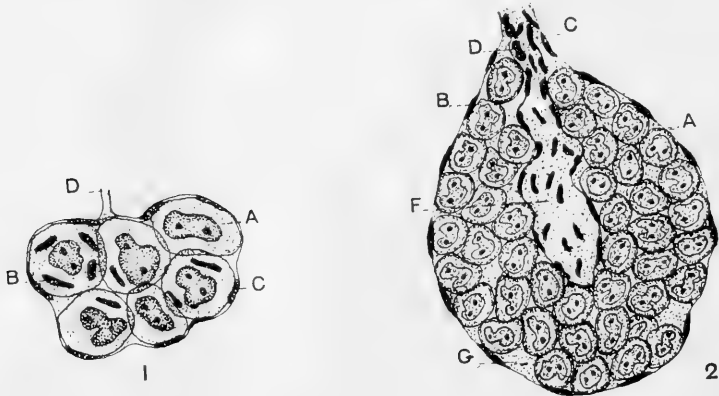
The gonads of the two series of tadpoles were sectioned at a thickness of  $7.5\ \mu$ . Heidenhain's iron hematoxylin stain was used exclusively. A counterstain of congo red was also employed, but equally satisfactory results were obtained without employing counter stains, especially in the young larvae.

Microscopic examination of the germ gland anlagen and germ cells of the two series of animals showed that the gross differences in size and somatic development, between fed and unfed larvae, did not extend to size or developmental differences in the germ cells. The germ cells of both control and starved animals were of the primitive, sexually undifferentiated type. A more rapid rate of cell division had evidently occurred in the algae-fed larvae, for the number of primordial germ cells in the gland was several times the number observed in the unfed larvae. The nuclear structure of the sex cells of the two series appeared

<sup>2</sup> This experiment started in spring of 1915; completion spring of 1916.

the same. Few yolk spherules were observed in the cytoplasm of the germinal cells of either group (figs. 1 and 2).

The structure of the germ glands, however, revealed considerably more differentiation in the control tadpoles than in the starved larvae. In the controls, the gonads were much larger than those of the unfed animals; the increased size was apparently due to a proliferation of the mesodermal cells into the anlage, together with an increase in the number of germ cells. In the gonads of the control animals, these mesenchyme cells had



All diagrams drawn to scale with the exception of figure 1.

Fig. 1 Germ gland of a larva starved twenty-six days from the date of development of the mouth. Series of May 6. A, primordial germ cell; B, yolk spherule; C, peritoneal cell; D, mesentery transverse section. Oil immersion objective used.

Fig. 2 Germ gland of control tadpole for animal shown in figure 1. A, germ cell; B, peritoneal cell; C, mesorchium; D, mesenchyme cell; E, primary genital space; F, secondary genital space.  $\times 800$ . Transverse section.

migrated into the gland by way of the mesentery and wedged themselves between the germ cells, completely investing some of them in clumps of three or four by delicate cytoplasmic strands, thus forming the germ cell nests which later become the cysts of the ovary and testis. The germ glands of the starved animals presented none of these changes, but instead, remained a simple, grape-like cluster of primordial germ cells, surrounded by a thin investment of peritoneum. No proliferation of mesenchyme cells into the gland had occurred (cf. figs. 1 and 2).

The larger size of the germ glands of the control animals is clearly shown in the text figures.

Sexual differentiation had not taken place in the starved larvae but had in the algae-fed controls.

At this stage of the work, considerable difficulty was experienced in determining the sex of the young tadpoles. The germ glands and germ cells of young frog larvae are so much alike in early stages that it is impossible to differentiate the sexes with certainty. Although there is a considerable mass of literature regarding the question of sex differentiation in the anurans, each investigator who has dealt with the problem seems to have set aside as untrustworthy the view of previous workers in the field, and to have each promulgated a new sex criterion of his own. I have found that only one or two of the many morphological features of the gonads advocated as sex criteria by the various authors, are trustworthy, when applied to very immature gonads.

The criterion of sex differentiation adopted for the smaller sizes of tadpoles was that advanced by King ('08) for *Bufo* and confirmed by Witschi ('15) for *Rana temporaria*. Both of these authors worked with young larvae, and found that in the germ glands of those animals destined to develop into males, the germ cells are scattered more evenly throughout the gland. Conversely, in the immature ovary which usually has a lumen, the germinal cells remain near the germinal epithelium, which is generally much thicker than in the males.

Conditions very similar to those described by King and Witschi for *Bufo* and *Rana temporaria* were noted in the larvae of *Rana pipiens*, and seemed to warrant the adoption of their criterion for sex differentiation in the larval stage of this amphibian.

May 15, another series of fed and unfed tadpoles was killed and sectioned. The nine day interval of starvation intervening between this series and that of May 6, had sufficed to bring about striking differences between the starved and control animals.

The algae-fed animals averaged 40 mm. in length; the largest animal of the lot attained a length of 46 mm. All of the controls showed marked growth and development of the hind limbs into their two primary divisions; also the toes had differentiated.

When compared with the control animals, the growth of the starved series of animals was found to be inhibited, for the average length of the unfed larvae was but 13 mm., approximately the same length as the tadpoles killed nine days before. No indications of limb development were observed.

Microscopic examination of the germ anlagen of the control and the starved animals revealed rather striking differences. The germ cells of the unfed tadpoles presented few developmental changes from those of the larvae killed May 6. The yolk spherules, present in the cytoplasm of the germ cells of the May 6 series, had disappeared. The number of germ cells appearing in transverse sections of the gland had increased slightly, showing that some multiplication of the germ cells had occurred.

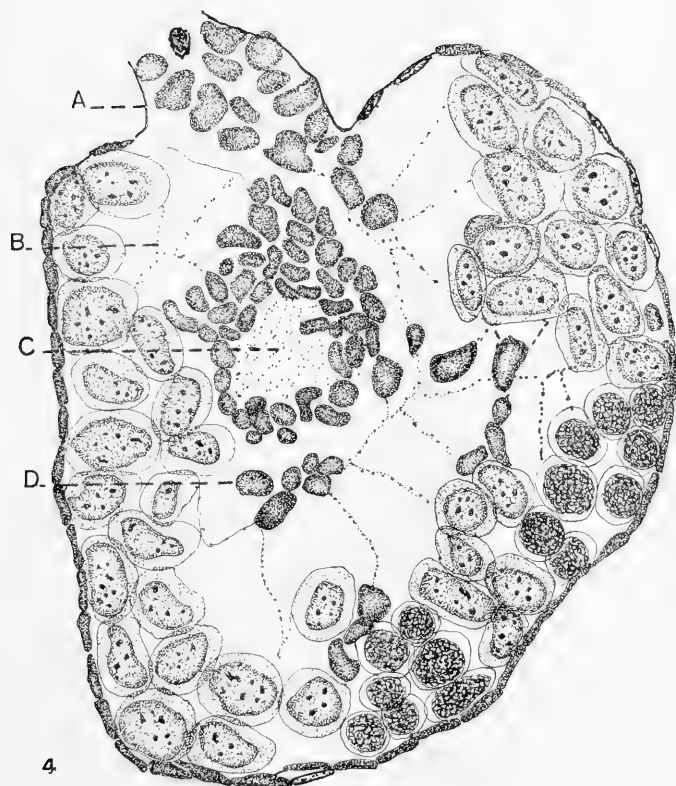
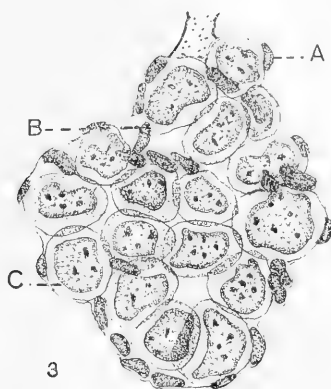
The structure of the germ anlage of the starved tadpoles had undergone no observable changes and remained small clusters of sexually undifferentiated germ cells (fig. 3).

Contrasted with the undifferentiated character of the germ glands of the unfed tadpoles, the gonads of the controls of this series presented marked developmental changes (fig. 4). The number of germ cells had greatly increased, there being many more cells in the gonads of the control (transverse section) as observed in the glands of the unfed tadpoles. This increase in the number of germ cells had greatly augmented the size of the glands, especially the length (transverse section). Many mesenchyme cells had proliferated into the glands forming a well developed secondary genital cavity. Sexual differentiation had occurred and in both male and female gonads the germ cells were partitioned off into nests or cysts by the migrating cells.

The germ cells of the control larvae presented striking nuclear changes, consisting in a rearrangement of the chromatin into the configurations characteristic of presynaptic stages. This was

Fig. 3 Gonad of tadpole starved thirty-six days. Series of May 15. *A*, peritoneal cell; *B*, mesenchyme cell; *C*, germ cell.  $\times 800$ . Transverse section.

Fig. 4 Gonad of control larva for animal shown in figure 3. Series of May 15. *A*, peritoneum; *B*, primary genital space filled with embryonic connective tissue; *C*, secondary genital space; *D*, mesenchyme cells.  $\times 800$ . Transverse section.



true only of the germ cells of female larvae. The male cells do not undergo the maturation process until several weeks after metamorphosis has occurred, and hence revealed no nuclear changes.

Mention has been made of the formation of a secondary genital space, and in the explanation of the text figures frequent reference is made to this cavity. A word of explanation might not be inappropriate as the term has been adopted from Kus-chakewitsch. This author explains his use of the term as follows:

In the development of the germ glands of frog larvae, two kinds of cavities appear—the primary and secondary cavities. The primary spaces are simply those vacant spaces appearing between the germ cells, which have never been obliterated by the migration of the sex cells into the gland and their subsequent multiplication. These primary spaces later become filled with embryonic connective tissue which, later in development, is directly transformed into the definitive connective tissue of the gonad.

The secondary genital space is the lumen formed within the mass of mesenchyme cells, which proliferate into the gland from the mesonephric region.

The next series of tadpoles from the control and starved cultures was killed May 25, ten days after the date of the last examination. The unfed animals averaged 14.5 mm. in length, showing an increase of length of 1.5 mm. in the ten day interval. This slight increase in length was probably due to the fact that, until a few days prior to the date of killing (May 25) the larvae had withstood the deleterious effects of prolonged starvation very well, aside from the fact that growth and development were inhibited. There was little mortality and no disposition on the part of the surviving tadpoles to feed upon their dead companions. About May 22 or 23, however, the mortality among the animals of the unfed culture greatly increased, so much so, in fact, that it became rather difficult to keep the containers free from dead larvae. The surviving animals, if left unmolested, would eagerly feed upon these. Only in this way is it possible to



account for the increase in growth of the tadpoles, for they received no food of any kind.

None of the starved animals revealed any indications of limb buds.

The control animals for this culture averaged in length 53 mm. All of the larvae possessed well developed hind limbs.

The structure of the germ cells and the germ glands of the fed and unfed animals presented interesting contrasts when examined microscopically. The germ cells of the starved larvae retained the primitive, undifferentiated character of primordial germ cells with this exception—that they contained no yolk granules. The size and average number of the cells in transverse section had not increased any when compared with the series killed ten days previous. No nuclear changes had occurred; nor had sexual differentiation taken place (fig. 5).

Contrasted with the germ cells of the starved cultures, those of the controls were far along in the developmental cycle. All of the germ cells were in synaptene or pre-synaptene stages in the larvae of the female sex. The germ cells appeared slightly larger than those of the unfed animals, but this is very likely due to the onset of the growth period of the young oocytes which occurs at the end of the synaptic stage. The average number of germ cells visible in transverse sections was very large as compared with the unfed animals of the same age (fig. 6).

June 7 another series of fed and unfed larvae was killed. The starved animals were, at this time, very sluggish, swimming about only when disturbed. The skin over the abdominal region appeared transparent, and through it, the shriveled viscera were plainly visible. The mortality among the starved larvae became so great at this time that it was found necessary to feed the tadpoles a few wisps of algae in order to prolong the experiment.

The starved animals when killed measured 15 mm. and a careful microscopic examination failed to reveal limb buds in any of them. Contrasted with the unfed animals, the controls were in advanced stages of metamorphosis. They averaged in length 60 mm., and had well developed fore and hind limbs. The tails of a few of the controls appeared frayed and ragged along the

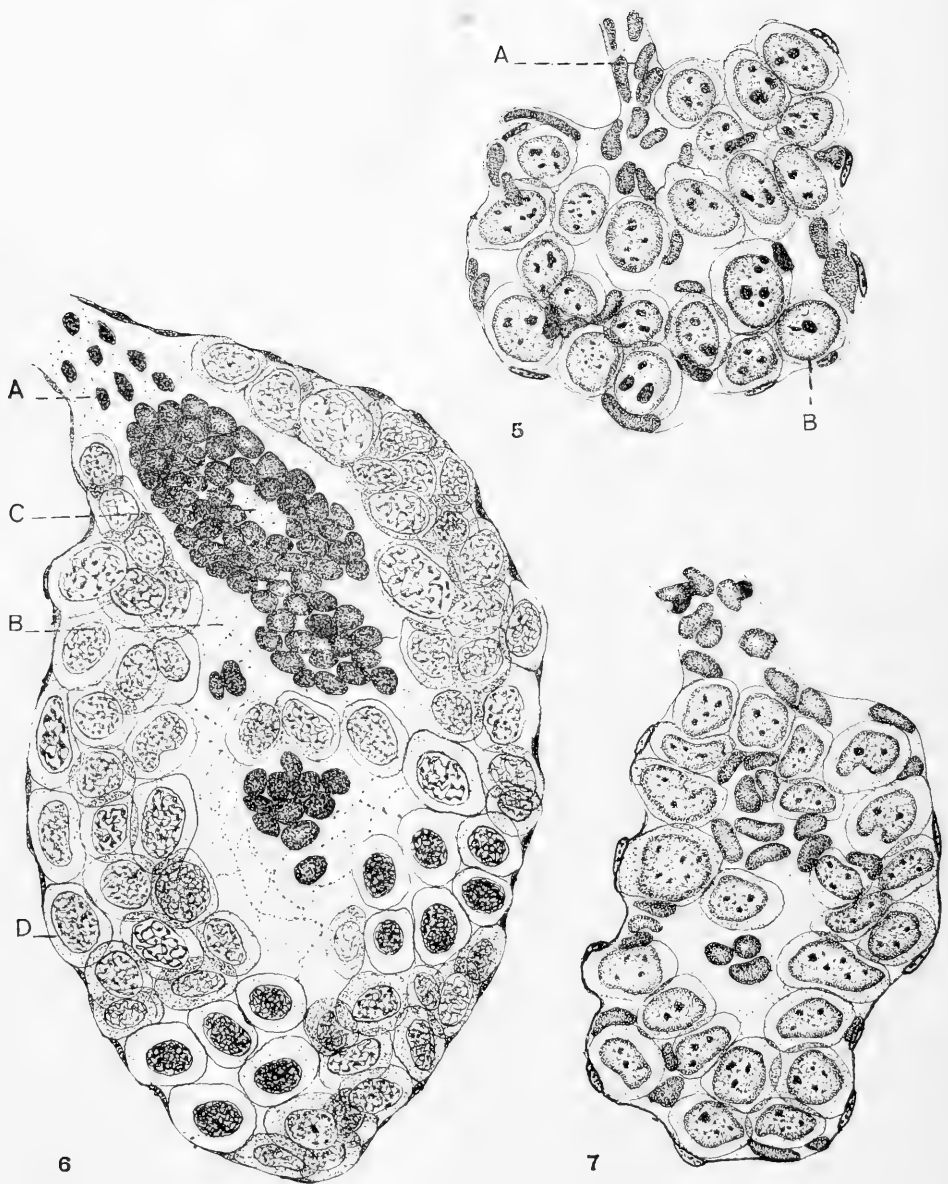


Fig. 5 Germ gland of larva starved forty-six days. Series of May 25. A, germ cells; B, mesenchyme cells.  $\times 800$ . Transverse section.

Fig. 6 Gonad of control for animal shown in figure 5. Series of May 25. A, mesenchyme cell; B, primary genital space; C, secondary genital space; D, germ cells.  $\times 800$ . Transverse section.

Fig. 7 Gonad of animal starved fifty-nine days. Series of June 7.  $\times 800$ . Transverse section.

edges, showing indications of atrophy and resorption. The sucker-like tadpole mouth was beginning to show signs of transformation to the typical frog mouth.

Microscopic examination of the sectioned material revealed little or no change in the germ cells and the germ glands of the starved tadpoles, other than those already recorded for the earlier series killed May 6, May 15 and May 25. The cells were still in the primitive, sexually indifferent state, and presented no nuclear changes indicative of maturation. No division figures were observed. The gonads were undeveloped though a few migrating mesenchyme cells were observed scattered through the glands (fig. 7).

As regards the control larvae, the number of germ cells had greatly increased and most of the cells of the female animals were in late synaptene or post-synaptene stages (fig. 8).

The germ glands of the controls were in advanced developmental stages (fig. 8).

The sex of the larvae was now easily distinguishable. The anlage of the females contained large cavities lined with endothelium. The number of migrating mesenchyme cells from the mesonephric tissue had greatly increased. The gross size of the gland had greatly increased; the large size being due chiefly to the connective tissue elements of the gland and not to any marked increase in the number of germ cells.

From June 1 on, the control animals began to metamorphose rapidly, and by June 17, the change from the larval to the adult state was complete in most of the larvae, except for a rudimentary tail.

The tail was completely resorbed in most of the animals by June 22. Owing to the difficulty of keeping the young frogs, and to the pressure of the other work, the control animals for the starved larvae were killed June 29.

The gonads of the young frogs had not, as yet, developed the morphological characteristics of these organs in adults. This is especially true of the male animals. In this sex, the transformation of the ribbon-like germinal mass lying on the ventral surface of the mesonephros into the oval shaped testes does not occur until several weeks after metamorphosis is completed.

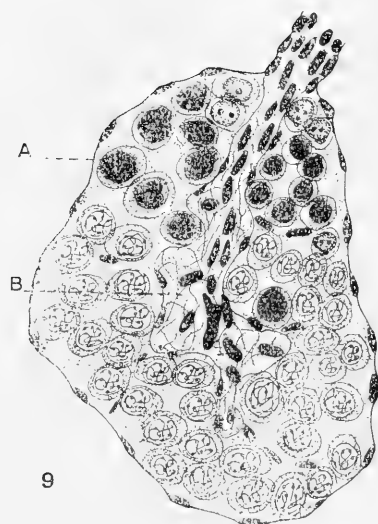
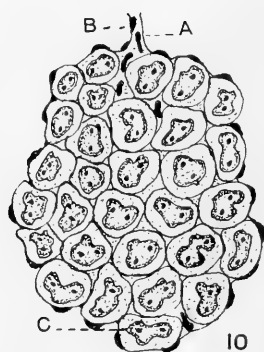
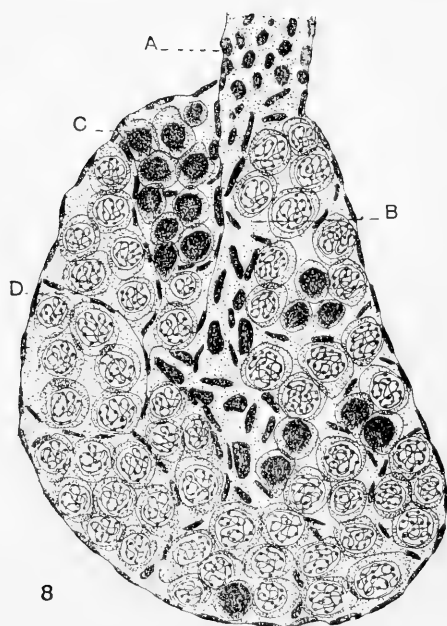


Figure 12 shows three oocytes from ovary of a young frog shortly after completing metamorphosis. Figure 13 is a diagram of three follicles from the testis of a young frog three weeks after metamorphosis.

July 8, one month after the last series of starved larvae of June 7 were killed, another series of unfed tadpoles was preserved for microscopic examination. The animals had been fed a few bits of algae three or four days previous to the date of killing. The animals were so weak and sluggish when examined that it was considered best to feed them bits of algae rather than risk their dying before the work was completed. At the time of death, the larvae averaged but 16 mm. in length and revealed no signs whatever of limb buds, whereas the controls for this series had completely metamorphosed by June 22, seventeen days previous.

The germ cells of this series of starved larvae were, for the most part, still of the primitive, sexually undifferentiated type (fig. 10). In one or two of the larvae, however, the germ cells appeared to be entering the early maturation stages. The chromatin had arranged itself into the thin spireme threads characteristic of presynaptic stages. The total number of germ cells within the anlage appeared to have increased somewhat as compared with the number observed in the germ glands of the unfed animals killed at earlier dates.

The germ glands of this unfed series of tadpoles were undeveloped, although the number of mesenchyme cells within the gland had increased somewhat when compared with the glands of previous series. Correlated with this increase in the number of mesenchyme and germinal cells, there was an increase in the

Fig. 8 Germ gland of control for animal shown in figure 7. Series of June 7. A, mesorchium; B, primary genital space; C, germ cells in synyzesis; D, ovarian cysts.  $\times 800$ . Transverse section.

Fig. 9 Germ gland of control larva from series of June 7. A, germ cells in synyzesis; B, embryonic connective tissue.  $\times 800$ . Transverse section.

Fig. 10 Germ gland of larva starved ninety-seven days. Series of July 8. A, mesenchyme cell; B, mesorchium; C, germ cells.  $\times 860$ .

Fig. 11 Germ glands of larva starved ninety-seven days. Series of July 8. A, germ cells in pre-synaptic stages.  $\times 800$ . Transverse section.

size of the gland, an increase greater than any revealed by other series of starved animals. None of the morphological criteria for sex differentiation were observed in the gonads of this series.

In the two larvae previously mentioned, i.e., those whose germ cells appeared to be entering the early maturation stages, the glands appeared to be male in character. The early maturation changes, however, are characteristic of female cells, previous to oocyte formation (fig. 11).

It seems anomalous that the germ cells of two of the unfed larvae should, in regard to development, be so far in advance of the gonad itself; for as was stated, many of the germ cells of two of the animals appeared to be in early maturation stages, whereas the gonads remained a simple cluster of cells. No differentiation of the gland had occurred. In all of the larvae heretofore examined, differentiation of the germ cells from the primitive, apparently sexually indifferent type, to the maturation stage, was correlated with marked development and differentiation of the germ gland anlage; the two conditions were apparently intimately related.

It may be that the two larvae whose germ cells presented such advanced developmental stages over the other animals of the same age and series, had succeeded in getting more food to eat than the other animals. Aside from a few filaments of algae now and then, however, these animals received nothing to eat.

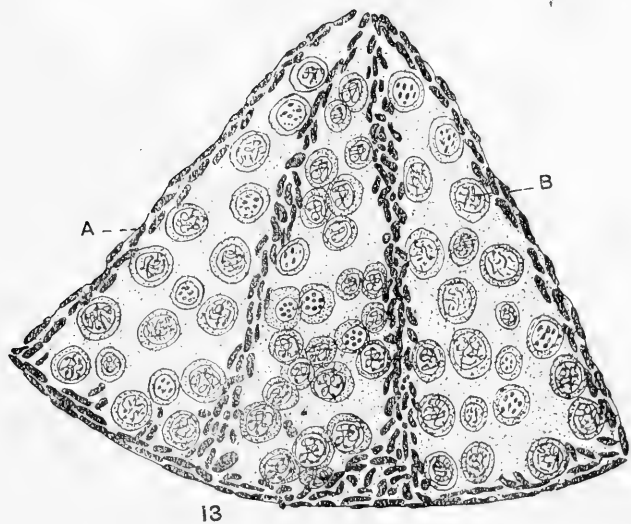
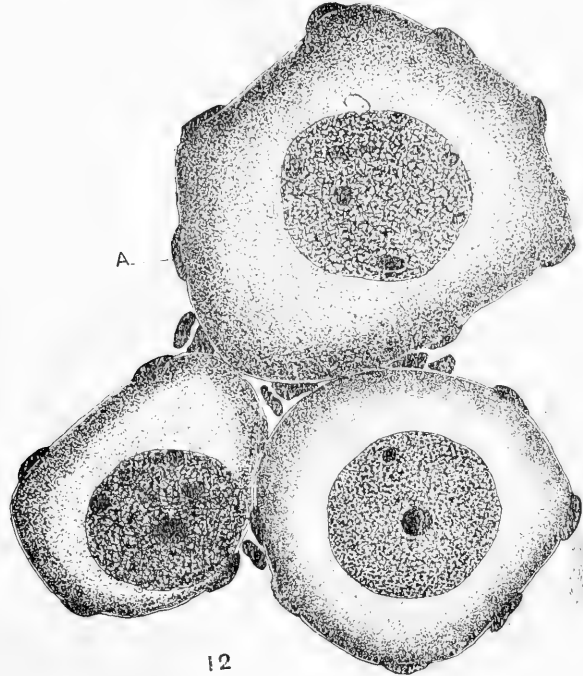
It may also be true that starvation inhibits the onset of the maturation processes of the germ cells for a definite time only, beyond which, its effects are not sufficiently potent to overcome the inherent tendencies of the sex cells to undergo differentiation.

It is unfortunate that more of the larvae of this series were not preserved for microscopic examination.

I succeeded in keeping three tadpoles of the starved culture until August 29, i.e., one hundred and thirty-nine days from the

Fig. 12 Oocytes from gonad of young frog of same age as animal shown in figure 11. A, Follicle cell.  $\times 800$ .

Fig. 13 Three seminiferous tubules from the testis of a young frog the same age as animal shown in figure 11. A, Interstitium; B, germ cells; in prophase stages of the first maturation division. A few sperm cells were observed.  $\times 800$ .



date the experiment began, many of the controls, as was mentioned before, had completed metamorphosis by June 22.

From July 8 on, however, the larvae became so weak and sluggish that it was absolutely necessary to feed them from time to time.

Every five days a few finely crumbled bits of bread were fed the tadpoles. This meagre diet, while sufficient to keep the animals



Fig. 14 Photograph of larvae starved one hundred and twenty-four days. The young frogs are the same age as the tadpoles. The shrunken appearance of the larvae is partly due to their being immersed in alcohol shortly before the picture was taken.

alive, was not nourishing enough to permit growth. The animals moved about the containers very feebly and only when violently disturbed. Most of the time they lay upon the bottom of the container.

These three tadpoles very likely could have been kept alive by underfeeding for an indefinite time, but unfortunately they were killed by accident, and at a time when it was impossible to pre-



serve them for microscopical examination. At the time of death, they were small tadpoles 15 mm. in length, and showed no indications of limb development or other signs of somatic development.

#### DISCUSSION

The experiment quoted apparently justifies the conclusion that total inanition not only prevents the formation of the germ anlage of *Rana pipiens* larvae, but inhibits the onset of maturation processes and increase in number of the germ cells.

It is an odd fact, but the germ cells of old larvae of a different species of frog are apparently much less affected by total inanition. A series of year old *Rana catesbiana* tadpoles, measuring 75 mm. at the beginning of the experiment, was starved for five months and ten days. The larvae received nothing to eat save perhaps the microscopic organisms found in all tap water. During this interval, the broad tails of the larvae shrunk to less than half their original width. The body musculature was partly absorbed and all growth and development was inhibited. Microscopic examination of the gonads revealed no observable differences between starved and control larvae. The large corpora adiposa of the controls, however, was so shriveled in the starved animals as to be almost unrecognizable; as this body is attached to the gonads, it may be that the germ cells derived their nourishment from it.

In view of Mead's ('00) work with starfish, in which he observed that sex is correlated with the attainment of a certain size (50 mm.) in these animals, the results obtained for the starved larvae of *Rana pipiens* are interesting. None of the small unfed tadpoles differentiated sexually. In the controls, sexual differentiation was first recognized with certainty when the tadpoles averaged 30 mm. in length. Very probably sex is recognizable earlier in this species when the animals are still smaller. No examination to test this point was made.

The observation that many of the germ cells of two unfed larvae, starved from the time of their emergence from the egg capsule (April 10) until July 8, were apparently entering early maturation stages, despite the fact that the development of the

soma and germ anlage had been completely inhibited, indicates that possibly the germ cells possess certain capacities for physiological recuperation from nutritional disturbances, independent of the soma.

It is more probable, however, that the two larvae received slightly more food than the other animals of the culture, and that their germ cells responded immediately by developmental changes to the stimulus of nourishment. The odd fact that the germ gland itself failed to respond is more readily understood when one takes into consideration the work of Nussbaum ('06). It was mentioned in the review of the literature early in this paper, that Nussbaum reported great shrinkage of the gonads of starved frogs, the glands shrinking to one-third their original size, but that when nourishment was given the animals, the gonads increased in size rapidly, whereas the remainder of the body remained emaciated. In view of these results, it appears probable that the germ cells of the two larvae in my cultures which were so far along in development, responded more readily to the slight food stimulus than the soma cells of the gonads; very likely the gonads would also have developed somewhat had the nourishment been greater.

#### SUMMARY AND CONCLUSION

1. Total starvation inhibits indefinitely the growth and the metamorphosis of larval frogs.
2. It prevents the development of the germ glands and delays any increase in the number of germ cells, interstitial cells and other tissue elements in the gonads.
3. Starvation greatly retards the normal cycle of development of the germ cells.
4. It prevents the onset of sexual differentiation.

I take this opportunity of acknowledging my indebtedness to Dr. B. M. Allen of the University of Kansas for suggesting this problem, and for the interest he has shown in the work.

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## EFFECTS OF THE EXTIRPATION OF THE THYROID GLAND UPON OSSIFICATION IN *RANA PIPIENS*

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TWO TEXT FIGURES AND THREE PLATES

### INTRODUCTION

The problem which is here described was undertaken at the suggestion of Prof. Bennet M. Allen, to whom I wish first of all to acknowledge my indebtedness for the incentive to this work and for much kindly assistance. I shall merely endeavor to discuss the effect of the extirpation of the gland upon the growth and development of the skeletal system, in the hope that this may be of some assistance to future workers who are interested in the effects of the removal of the glands of internal secretions.

### LITERATURE

It has been shown in a number of papers that have appeared during the last few years, that the removal of the thyroid gland in rabbits, pigs, cats, dogs and even man produces a marked retardation in the development of the skeletal system.

Hofmeister ('92) found that the extirpation of the thyroid of young rabbits, the external parathyroids being left 'in situ' was followed by a retardation of growth and the development of a condition of chronic cachexia. Further experiments show that the arrest of growth was due to a remarkable retardation of the process of ossification, both of the epiphyses and of the synchondroses. The long bones, the pelvis and the vertebral column, showed the greatest departure from the normal, the skull the least. The growth of the long bones was retarded to the extent of at least a third of their proper length, and micro-

scopical examination of the epiphysial line showed specific degeneration, consisting in a reduction of the normal cell proliferation, combined with vesicular swelling of the cartilage lacunae and shrinkage or even partial destruction of the cells.

Eiselsberg ('95) found that in sheep whose thyroid had been removed at the age of ten days there was a conspicuous retardation of growth and a high degree of derangement of the processes of skeletal growth. In goats operated on three weeks after birth the same author observed shortening of the bones of the extremities, marked shortenings of the frontal part of the head, the occipital part appearing exaggerated by comparison. Similar results have since been obtained with dogs by Massaglia ('07) and by Parhon and Goldstein ('09) with cats. So it seems just to conclude that the arrest of growth of the skeleton is the typical and invariable result of the absence of the thyroid function in both carnivora and herbivora. The most constant symptom says V. Bruns<sup>1</sup> in man as well as in animals is a remarkable disturbance of growth. The arrest of development produces a dwarf like appearance of the individual due to imperfect endochondral ossification. The enclosed epiphysial line may be seen in skiagrams and post mortem examination shows that the ossification of the ends of the epiphysis is extremely deficient while the periosteal bone formation is undisturbed. So we see that a comparatively large amount of work has been done upon the effects of the removal of the thyroid gland in mammals and even its effects have been studied to a greater or lesser extent in man, but up to this time no attempt has been made to work out the effect upon the development of the skeletal system produced by the removal of this gland in Amphibians. An excellent review of the results and interpretations of various workers has been given by Biedl in his recent book on the "Internal Secretory Organs," so that it will be unnecessary to devote any more space here to the discussion of the general problem.

<sup>1</sup> Quoted from Biedl; Internal Secretory Organs.

## MATERIAL AND TECHNIQUE

The material from which these observations were made was collected near Lawrence, Kansas, during the early spring of 1916 and the thyroid gland was removed by Professor Allen when the *Rana pipiens* were about 6 to 6.5 mm. in length. About one half of the number collected were operated upon and the remainder were kept as control specimens.

The metamorphosis of the control specimens began about the middle of July and continued until August 13, while the operated tadpoles failed to metamorphose and continued to live on in their larval state maintaining largely their larval characteristics. These specimens both thyroidless and normal controls have been killed at various times ranging from July 25, 1916 to March 28, 1917, so as to trace the development of the skeletal system as clearly as possible.

As to the method of study I may justly divide it into three parts. First, a study of whole mounts, cleared by the Schultze caustic potash glycerine method; second, a microscopical study of sagittal and transverse sections of the vertebral column; and third a study of the cartilage and bones of the hind legs stained 'in toto' by means of the Lundvall method.

In the first method the whole mounts are prepared, after measurements have been taken and fixation has been accomplished, by first placing the entire specimen in a two per cent solution of potassium hydroxide. It is left in this solution for about eight to ten days, after which it is placed in a twenty per cent glycerine solution for clearing where it is kept for about two days; it is then placed in a stronger solution for an equal length of time and so on until a full strength glycerine solution is reached where the whole mount may be studied and kept indefinitely. In specimens which have thus been treated we find that the cartilage appears transparent while the bone appears more or less opaque.

Coming now to the second method, viz., that of a microscopical study of sagittal and transverse sections, which if any distinctions are to be made between the methods of study,

must be considered as the most important of the three, consists in the removal and sectioning of the vertebral column of both thyroidless and normal control specimens. The fixations used were Flemming's solution, formalin and alcohol. All of these gave excellent results but on the whole the Flemming material was the most satisfactory. After the material was fixed it was placed in a two per cent acetic acid solution for decalcification. At first, the material was cut in sagittal sections and an attempt was made to work out the various parts of the vertebral column, but this soon proved to be more or less unsatisfactory because of difficulty of interpretation, so it has later proved more satisfactory to cut the material in transverse sections. By this method there is no chance of misinterpretation of parts and the orientation of the section is easily accomplished.

The sections were all cut ten to fifteen micra in thickness and the staining was done either with Meyer's haemalum or Heidenhain's iron-haematoxylin, an alcoholic solution of eosin being used in both cases as a counter stain.

The third and last method of study, viz., the staining of the cartilage and bone 'in toto' by the Lundvall method is especially useful in studying the bone and cartilage formation of the hind legs. The method is as follows, four parts of a saturated solution of alizarine crystalizatum in 95 per cent alcohol was mixed with one part of a very weak solution of either methyl blue or toluidin blue in 95 per cent alcohol. Specimens to be stained were placed in the stain and kept in a thermostat at 32°C. for from one to several days. They were then run up through the alcohols and finally cleared in a solution of benzol benzoate which must be so mixed that the index of refraction of the mixture is the same as the index of refraction of the tissue. In specimens which have been stained by this method the bone takes on a red color while the cartilage takes on a sky blue color.

#### RESULTS

The results of this work are based upon a study of twenty-seven specimens, of which number twelve were thyroidless and the remainder were controls.



The first indication of the effect of the removal of the gland is shown by the fact that the tadpole fails to metamorphose and maintains its larval characteristics after it has passed the period when the change should have taken place. The head becomes flattened dorsoventrally, the tail fails to shorten, and the hind legs are greatly delayed in development. In tadpoles three months past the period of metamorphosis the hind legs have reached a length of only 4 to 5 millimeters all of which clearly goes to show that the removal of the gland has produced a very great effect upon the development of the body beginning either at or probably before the period of metamorphosis. A complete account of the external characteristics of growth has been given in the previous article by Allen

Before attempting to make a comparison of the deposition of the calcium salts in the thyroidless and control specimens let us consider the normal process of deposition. What is the nature of the salts, what part do they play in bone formation and what relation do they bear to the centers of ossification? In the normal control animal the process of the deposition of the calcium salts begins at three points in the vertebra, viz., the centrum, neural arch and transverse process. These are known as deposition centers and later become centers of ossification. The first change in the cartilage takes place in these centers, the cartilage cells increase in size and in number in such a way that several enlarged cartilage cells come to lie in a single enlarged cell space. The cells now arrange themselves in rows, there is an increase in the intercellular matrix and a deposition there of calcium salts. By this process the cartilage becomes calcified and the areas involved are known as calcification centers.

Ossification proper begins by blood vessels from the periosteum pushing their way into the calcified cartilage at the calcification centers carrying with them some of the osteogenic tissue from beneath the periosteum. The blood vessels carrying this tissue are known as periosteal buds. Osteoblasts now develop from the osteogenic tissue and dissolve the calcified cartilage. Free hand sections cut through the vertebrae show

that the osteoblasts are first formed in the center of the neural arch and it is here that the first dissolving away of the salts takes place. As the process continues they gradually work their way toward the outside of the vertebrae until they reach the periosteal bone where the process appears to stop.

In making a comparison of the thyroidless and control animals let us first consider figures 1 and 2. These represent end views of the third vertebrae. Figure 1 represents the vertebra of a normal control frog killed August 6, body length 21.5 mm. and figure 2 represents the vertebra of an operated tadpole killed October 14, total length 80 mm., body length 29 mm. In comparing these two individuals one first notices the difference in size of the two vertebrae and also a great difference between them in the extent of the calcification of the cartilage in the centrum and neural arch regions.

In comparing these two it is seen that the vertebra of the thyroidless animal is considerably larger than that of the control, but this increase in size is no doubt due to the fact that the thyroidless animals have continued to grow in size after ceasing to differentiate and are actually larger than the normal tadpoles at the time of metamorphosis. In comparing the centra of the two individuals we find the most striking difference. The centrum of the thyroidless tadpole killed October 14, is more than a third larger than is the centrum of a control killed August 6, while actual measurements show that the percentage of deposition of calcium salts of the control compared with that of the thyroidless specimen is as 5 to 9. This numerical comparison is made by taking an average of the thickness of the calcified portions of the centrum.

In the metamorphosed control there has been a marked degree of absorption of the calcified cartilage.

In comparing the thyroidless tadpole which was killed March 6, total length 102 mm., body length 40 mm. with the control frog killed March 28, body length 56 mm. we find that there has been continued growth of the vertebrae of the normal frog until they have outstripped those of the thyroidless tadpoles. This is in

correspondence with the fact that the former have outstripped the latter in body length.

Another very interesting phase in comparing the vertebrae of the thyroidless and control tadpoles is the nature of the deposition of the calcium salts (figs. 1, 2, and 5). Figure 5 represents the third vertebra of a normal control tadpole killed before metamorphosis, total length 52 mm., body length 23 mm. when the hind legs have reached a length of only twelve millimeters. Especially to be noticed at this early stage is the uniformity of the deposition of calcium salts throughout the neural arch and the centrum. In the control frog killed August 6 (fig. 1), body length 21.5 mm. the vertebra appears quite different, the cartilage cells have been invaded by the osteoblastic tissue and a large part of the calcium salts have been eroded away or completely absorbed. The centers of deposition appear more plainly marked than in the earlier stages and are especially noticeable near the ends of the neural arch as we approach the neuro-central suture and the dorsal bridge. Heavier deposition is also noticed in the centrum and in the transverse process some distance out from their point of union with the neural arch. The same irregularity of deposition as was noticed throughout the vertebra is also to be observed in the centers of deposition. In other words there is a clumping together of the calcium salt spicules giving us what we might term small centers of deposition within larger centers. The same thing is true of the centrum. Its outline is more or less irregular, the deposition is not uniform, and it is confined almost entirely to the ends and lateral portions as clearly shown in figures 1 and 3. The dorsal and ventral sides contain a very small amount of calcium salts and are easily shattered in free hand sectioning. The most striking thing in the case of the controls is the deposition of salts lateral to the centrum which is persistent in all forms studied after the time of metamorphosis and which is not at all shown in the thyroidless tadpoles. In the thyroidless animal killed February 7, total length 123 mm., body length 43 mm. we see that a great part of the neurocentral suture has been filled in by deposition lateral to the neural

arch rather than by deposition lateral to the centrum as was the case in the controls above mentioned.

In comparing the thyroidless tadpole at the time when metamorphosis should normally take place with a normal control of the same age we see that the amount of calcification in the one corresponds very closely with that in the other, but especially to be emphasized at this stage is the fact that the process of ossification in the thyroidless animal is greatly delayed. The cartilage cells appear to be almost completely calcified but the process of absorption of these salts appears to be almost stopped. The same spicular arrangement of the granules can be observed but they are more or less fused together and as a result they appear more uniform in their distribution. The centers of deposition have a very definite and regular outline and are clearly marked off from the rest of the vertebra (figures 2 and 4). Further differential points observed were the presence of a heavy deposition of salts in the rib and their uniform distribution in the centrum. One also notices in the operated tadpole killed October 14, total length 80 mm., body length 29 mm. that there is an absence of deposition lateral to the centrum and that the line of separation between the neural arch and the centrum is very definite.

In the specimen killed seven months past the period of metamorphosis (fig. 6) I have failed to observe any striking changes over the specimen above described other than a gradual development of the vertebra consisting of an increase in size, an increase in the amount of calcification and a partial filling in of the dorsal bridge and the neuro-central suture. The neuro-central suture is almost closed in the first and second vertebrae especially on the anterior side, while the length of the dorsal bridge has been greatly reduced. The centers of deposition are still persistent but are much less prominent due to the heavier deposition of salts in the other parts of the vertebra.

The next point of interest is a comparison made in the dorsal bridge region of the vertebra. In the case of the operated tadpoles the development of the cartilaginous bridge is greatly delayed. In the normal control tadpoles the spinous process

develops very early in the formation of the vertebrae. Figure 5 shows that its cartilaginous anlage is well developed even at the time of metamorphosis and probably before. In the control frog killed March 28 (fig. 9), not only the lateral portion of the neural arch, but the dorsal bridge as well is completely ossified and the primitive cartilage cells have been replaced by endochondral and periosteal bone.

In the thyroidless tadpole killed Oct. 14, total length 80 mm., body length 29 mm. not even the rudiments of a spinous process are developed, and even in specimens which are seven months past the period of metamorphosis it is not yet evident. Comparison of the cartilaginous bridges shows that the control is in a more advanced stage. The cartilage of the operated tadpole is of a very primitive hyaline type and appears merely as a gelatinous mass. In specimens killed four months past the period of metamorphosis it is in a somewhat more advanced stage but the amount of calcification is almost negligible. Measurements of both operated and control specimens which were killed two to three months after metamorphosis fail to show any marked differences in the width of the dorsal bridge.

#### MICROSCOPICAL STUDY

Now that it has been shown through the study of whole mounts that there is a marked difference between the deposition of calcium salts in the thyroidless and control specimens, it becomes of interest to know their histological nature.

After a careful study of the vertebral column of the thyroidless and control animals it was found that the third vertebra might be taken as a typical example and it is for this reason that reference will be made to this one in particular.

Histological study of the vertebral column of a normal control tadpole shows that the process of ossification begins very early. Figure 13 represents the amount of ossification present when the hind legs have reached a length of only twelve millimeters. At this early stage of development very little endochondral ossification of the centrum was noticed but the ossification of the neural arch was well under way. Figure 9

represents a reconstructed drawing of the third vertebra of a normal control frog killed March 28. In this individual we see that we are dealing almost entirely with periosteal bone and osteoblastic tissue. The numerous cartilage cells present in the earlier stages (fig. 13) have been almost completely broken down and the few cartilage cells that remain are only to be found at the ends of the rib and in the region of the centrum. There no longer exists a definite neuro-central suture and dorsal bridge but they have both become a part of the neural arch and the primitive cartilage cells present at the time of metamorphosis have been replaced by bone.

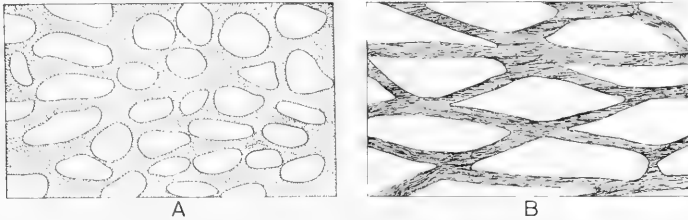
In the thyroidless tadpole there is a great retardation in the process of ossification and the operated animal killed October 14 compares very closely with the control killed before metamorphosis (fig. 12 and 13). In the operated tadpole killed February 7 (fig. 10 reconstructed) the periosteal bone is very poorly developed and we are dealing almost entirely with a mass of hyalin cartilage cells which in the centrum have been invaded only in a few places by endochondral bone spicules. The line separating the centrum and the neuro-central suture is very plain and the cartilage cells of the latter are of a primitive hyalin type. There also appears to be a marked retardation in the development of the periosteal bone, it appears as a very thin layer surrounding the outside of the arch and diffuses into the hyalin cartilage cells in the region of the dorsal bridge.

In our study of the whole mount of the operated tadpole killed February 7 we especially emphasized the fact that the width of both the neuro-central suture and the dorsal bridge had been greatly reduced. Microscopic examination shows that both the neuro-central suture and the dorsal bridge are much wider than was previously interpreted. The cartilage cells appear to have reached a comparatively late stage in calcification but as yet no sign of ossification is present.

Figures 15 and 16 represent cross sections through the neural arch and serve merely to show further the great retardation in the process of ossification in the operated specimens.

Let us now compare the operated tadpole killed October

14 with the one killed March 6 (figs. 12 and 14). During the period of five months which has elapsed there appears to have been very little change in the process of ossification. The centrum and neural arch of the operated tadpole killed March 6 compared with the one killed October 14 shows no marked advance in ossification and as previously stated compares very closely with the control killed before metamorphosis. It is quite true that there has been somewhat of an advance in the process of calcification, for example, the width of the neuro-central suture and the dorsal bridge have been considerably decreased in this later stage and the cartilage cells in this region



Text fig. A Transverse section through the intervertebral disc of a normal control frog killed March 28 showing the character of the hyalin cartilage cells.

Text fig. B Transverse section through the intervertebral disc of a thyroidless tadpole killed March 6, total length 102 mm., body length 40 mm. showing the character of the fibrous cartilage cells.

show a somewhat more advanced stage in their development but this advance is very slow indeed. In comparing the control killed before metamorphosis and having a leg length of 12 mm. with the normal frog killed March 28 one observes striking changes. The dorsal bridge and the neuro-central suture have become a part of the neural arch, the primitive cartilage cells have been transformed into bone, ossification has continued in its normal course and has almost reached a stage of completion. So it seems fair to conclude that the removal of the thyroid has produced a great retardation in the process of ossification, that amounts almost to a cessation of the process. Another interesting point is shown by a study of the cartilage cells of the intervertebral disc (text figs. A and B). In case of the operated tadpole the cartilage is of a very primitive fibrous type while that

of the control is of a late fibrous or early hyalin type. Our study would therefore seem to indicate that the removal of the thyroid gland has produced a general retardation in the process of ossification which is not confined to any one part of the vertebral column in particular.

#### STUDY OF THE HIND LEGS

A study of both thyroidless and control individuals shows that the same principles which were applicable to the bone formation of the vertebral column are likewise applicable to the bone formation of the hind legs, and especially is it to be emphasized that the retardation of ossification in the bones of the hind legs is much more marked than in the case of the vertebrae. Even so great is the retardation that one is almost justified in saying that the process of ossification after it has reached a certain stage is completely stopped.

The results can best be studied by referring to figures 17 and 18. Figure 18 represents the 12 mm. hind legs of a normal control tadpole killed before metamorphosis while figure 17 represents a 4.5 mm. hind leg of an operated tadpole killed October 14.

In the control tadpole having a leg length of 12 mm. we find that the bones of the hind legs are completely formed although still in a very early cartilage state. The tibia-fibula is joined only at the epiphysis. The centers of ossification are clearly marked and the osteogenic tissue has invaded the cartilage at the extremities of the bones and has given rise to the well marked epiphyses.

In the operated tadpole we have only three marked centers of ossification present, and these are found in the femur, tibia and fibula. The tibia and fibula are completely separated as is also the astragalus and the calcaneum due to the absence of the epiphysis at this early stage. Only four of the digits are present and these are represented by only one bone in each with the exception of the fourth which has two.

In comparing the 5 mm. hind leg of the thyroidless tadpole killed March 6 with the 6 mm. hind leg of a normal control



animal we see several marked differences. In the thyroidless animal we have again only the three centers of ossification present, but in the control in addition to these three centers we also find ossification taking place in the calcaneum and astragalus bones. The bands of ossified bone are considerably larger, and the digits are almost perfectly formed at this early stage. So again it seems just to conclude that the removal of the thyroid gland has greatly retarded if not completely stopped the process of ossification as well as the process of growth.

#### SUMMARY

##### *1. Control tadpoles*

The process of calcification is almost complete by the beginning of metamorphosis. By the time metamorphosis is completed the process of ossification has proceeded so far that the calcified cartilage has become markedly reduced by absorption. The process of ossification continues until by March 28th the neuro-central suture has become almost obliterated and both periosteal and endochondral bone are well developed. There is, however, at that time a total absence of ossification in the ribs. The spinous process is well developed by the time of metamorphosis, but calcification does not appear in it until later.

##### *2. Thyroidless tadpoles*

The vertebrae of the thyroidless tadpoles continue to increase in size long after the period when the controls have metamorphosed. Although the vertebrae are strictly larval in character, both in form and as regards their very early stage of ossification, nevertheless they continue to grow in size in correspondence with the growth of the body, becoming much larger than the vertebrae of the controls that had undergone metamorphosis; but retaining characters far more primitive than those shown by the latter. This is especially seen in the absence of the spinous process even in the tadpole killed March 6. The cartilage becomes extensively and rather uniformly calcified, invading a large portion of the neural arch. The

centers of calcification remain distinguishable for some time. The neuro-central suture becomes almost entirely filled in by calcification. There is a heavy deposition of calcium salts in the end of the rib. The cartilage of the dorsal bridge and of the neuro-central suture is of a primitive hyaline type.

The most striking feature of the vertebrae of the thyroidless tadpoles is the almost complete absence of ossification. While the normal controls clearly show both periosteal and endochondral ossification at the time of metamorphosis, the thyroidless tadpoles show only the faintest indications of these processes, even 7 months after the metamorphosis of the controls. This in spite of the fact that they have continued to grow far beyond the size attained by the latter at metamorphosis.

### *3. The limbs in control and thyroidless tadpoles*

The removal of the thyroid gland has greatly retarded, if not completely stopped both the process of ossification and the process of growth in the bones of the hind legs.

It is thus seen that while calcification of the cartilage has proceeded in the absence of the thyroid gland, there has been an extreme retardation in the process of ossification.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Surface view of the third vertebra of a normal control frog killed August 6, body length 21.5 mm. showing the irregular deposition of the calcium salts, the deposition of calcium salts lateral to the centrum and the well developed spinous process.

2 Surface view of the third vertebra of a thyroidless tadpole killed October 14, total length 80 mm., body length 29 mm. showing the uniform deposition of calcium salts, the well marked centers of calcification, the presence of a deposition of calcium salts in the rib, the total absence of a spinous process, and the regular outline of the calcium salts in the centrum.

3 Surface view of the third vertebra of a normal control frog killed August 6, body length 21.5 mm. showing the heavy deposition of calcium salts on the sides and ends of the centrum, and the deposition of salts lateral to the centrum filling in the neuro-central suture.

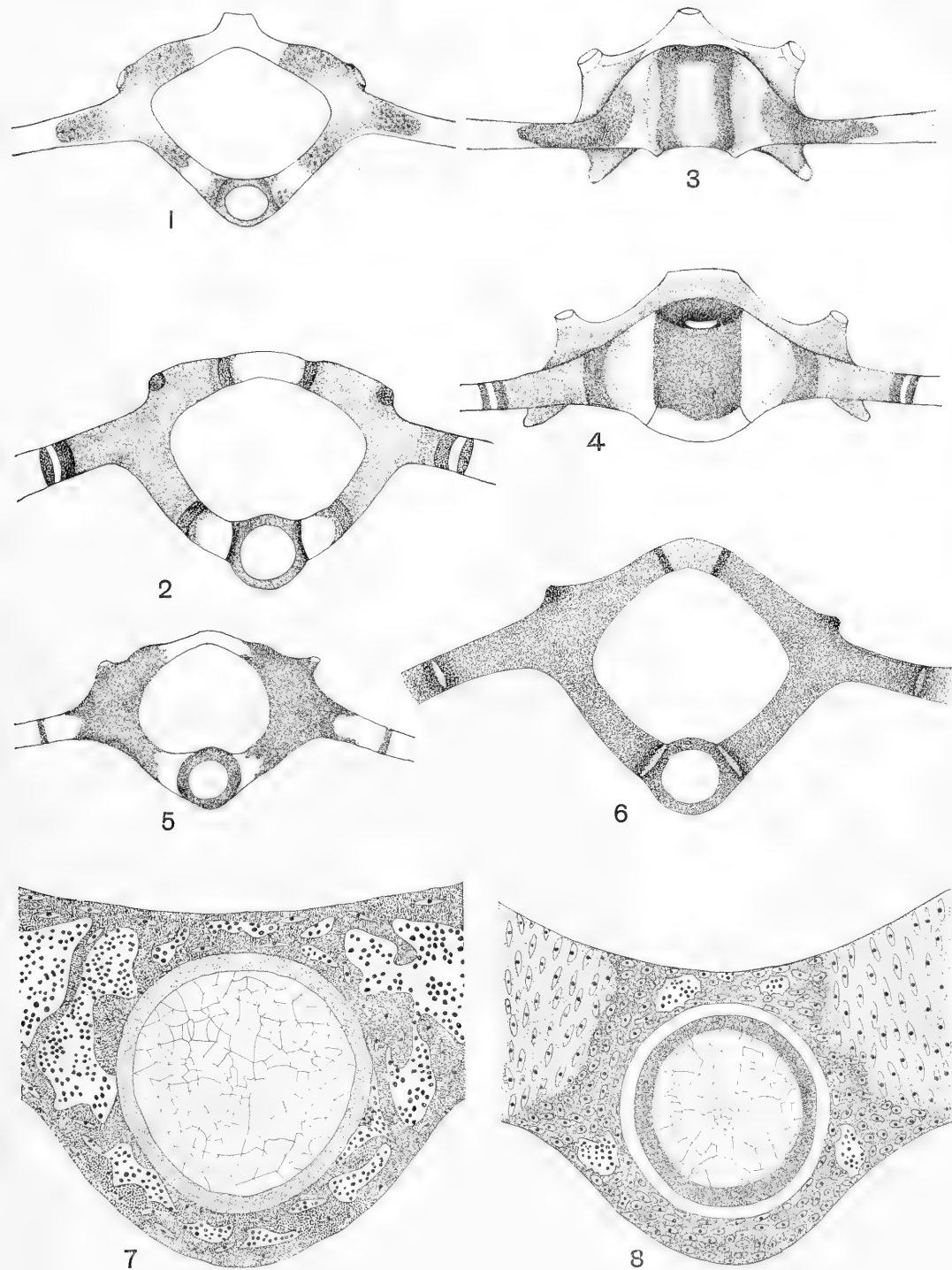
4 Surface view of the third vertebra of a thyroidless tadpole killed October 14, total length 80 mm., body length 29 mm. showing the heavy deposition of calcium salts in the centrum, the marked centers of calcification and the lack of deposition lateral to the centrum.

5 Surface view of the third vertebra of a normal control tadpole having a leg length of twelve millimeters, total length 52 mm. showing the uniform deposition of calcium salts in the centrum and neural arch before metamorphosis and also the fairly well developed spinous process.

6 Surface view of the third vertebra of a thyroidless tadpole killed February 7, total length 123 mm., body length 43 mm. showing the increase in size, the increase in the amount of calcification and the partial filling in of the neuro-central suture and the dorsal bridge.

7 Transverse section of the centrum and neuro-central suture of a normal control frog killed March 28, body length 56 mm., showing the large amount of periosteal bone and osteogenic tissue.

8 Transverse section of the centrum and neurocentral suture of a thyroidless tadpole killed March 6, total length 102 mm., body length 40 mm. showing the absence of the periosteal bone formation, the early stage of the hyalin cartilage cells of the neuro-central suture and the small amount of endochondral ossification in the centrum.



## PLATE 2

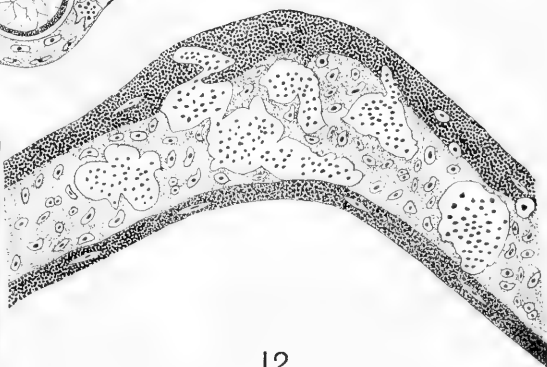
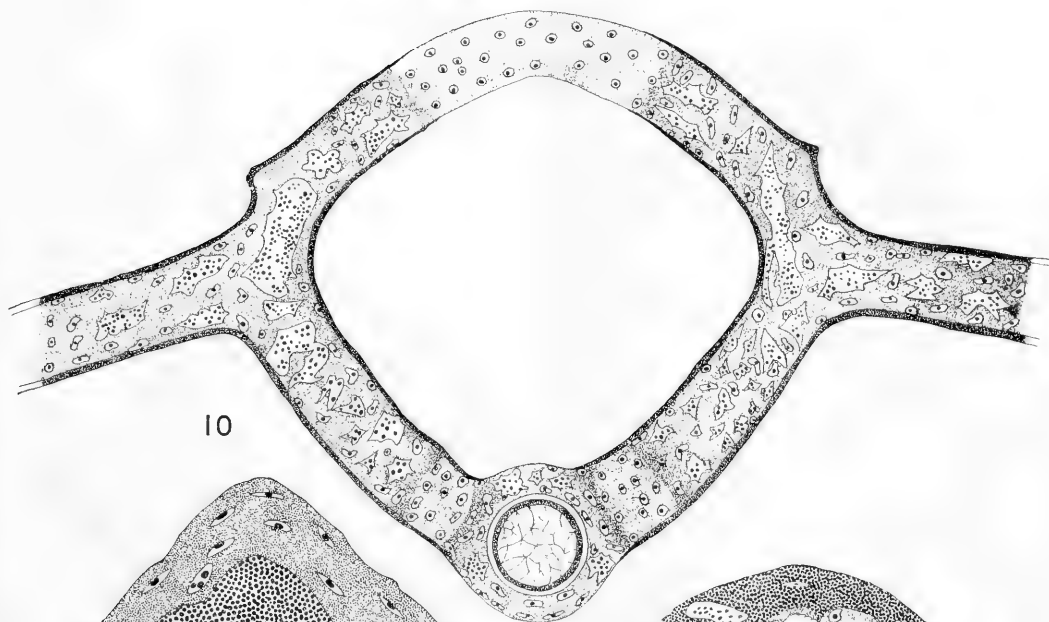
### EXPLANATION OF FIGURES

9 Reconstructed Transverse section of a vertebra of a normal control frog killed March 28, body length 56 mm. showing the great amount of periosteal bone and osteogenic tissue.

10 Reconstructed Transverse section of the vertebra of a thyroidless tadpole killed February 7, total length 123 mm., body length 43 mm showing the poorly developed periosteal bone, the small amount of endochondral ossification and the primitive condition of the cartilage cells of the dorsal bridge and neuro-central suture.

11 Transverse section through the neural arch of a normal control frog killed March 28, body length 56 mm., showing the well developed periosteal bone and the large mass of osteogenic tissue.

12 Transverse section of a thyroidless tadpole killed March 6, total length 102 mm., body length 40 mm. showing the poorly developed periosteal bone and the comparatively small amount of endochondral ossification.



### PLATE 3

#### EXPLANATION OF FIGURES

13 Transverse section of the neural arch of the vertebra of a normal control tadpole killed before metamorphosis, total length 52 mm., body length 23 mm. The legs have reached a length of only 12 millimeters. This shows the amount of endochondral ossification present.

14 Transverse section of the neural arch of the vertebra of a thyroidless tadpole killed October 14, total length 80 mm., body length 29 mm. showing the amount of endochondral ossification present.

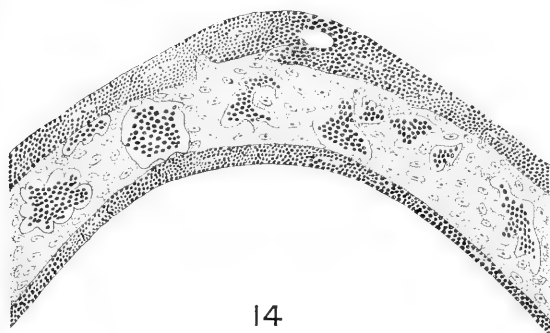
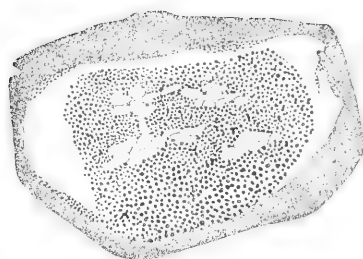
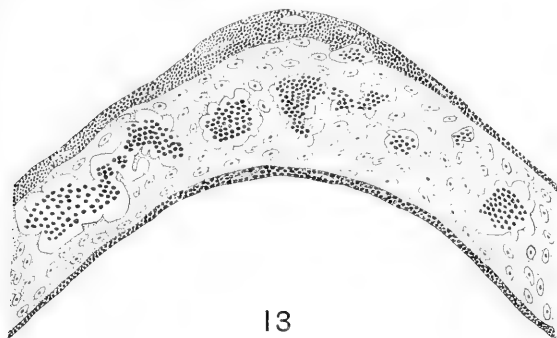
15 Cross section through the neural arch of the vertebra of a normal control frog killed two months after metamorphosis, showing the periosteal bone and osteogenic tissue.

16 Cross section through the neural arch of the vertebra of a thyroidless tadpole killed December 15, total length 52 mm., body length 23 mm. showing the periosteal bone formation and the endochondral ossification.

17 Surface view of a four and one half millimeter hind leg of a thyroidless tadpole killed October 14, total length 80 mm., body length 29 mm. showing the cartilage and bone formation.

18 Surface view of a 12 mm. hind leg of a normal control tadpole total length 52 mm., body length 23 mm. showing the cartilage and bone formation.







## THE EFFECT OF THE EXTIRPATION OF THE THYROID UPON THE THYMUS AND THE PITUITARY GLANDS OF *RANA PIPIENS*

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ONE PLATE AND TWO CHARTS

This problem was worked out in the laboratory of the Zoology Department of the University of Kansas under the direction of Dr. B. M. Allen in connection with other problems concerning the experimental removal of glands of internal secretion from *Rana pipiens*. Due credit is given for advice, material, and assistance, all of which have been of great help.

The thymus and the pituitary glands of a number of specimens of *Rana pipiens* in different stages of development were measured and drawn with camera lucida and comparisons made with thyroidless specimens.

The thymus is a paired gland found on the sides of the head of the frog just posterior to the tympanic membrane. In section the thymus appears as a round, compact mass of deeply staining cells with a darker outer portion and an inner part that stains somewhat lighter. The characteristic thymic corpuscles are found in the frog thymus. The general appearance is that of lymphoid tissue but it is characterized by the thymic corpuscles. The thymus usually degenerates when sexual maturity is reached but may last through the life of the individual. The thymus is now considered as of lymphoid character but with the function of an organ of internal secretion that has to do with normal growth and sexual development. The thymic corpuscles are interpreted by some as the portions that have the secretory function (Bell '05).<sup>1</sup> It has been regarded as the original source of the leukocytes (Beard

<sup>1</sup> Bell, E. T. 1905. *Am. Jour. Anat.*, vol. 5, pp. 29-62.

'00).<sup>2</sup> This theory however is not generally accepted. There is much controversy as to the thymus being essential to life. Some writers say it is not but that its removal has an effect upon the sex glands. Gudernatsch ('14)<sup>3</sup> says that thymus feeding increased the growth, but retarded differentiation in frog tadpoles.

The material was furnished by Prof. B. M. Allen and was used by him in his experiments upon the removal of the thyroid.

The controls consisted of a number of tadpoles raised under the same conditions as the thyroidless and a number of normal frogs in various stages before, during and after metamorphosis.

In the stages up to and including the 24 mm. stage the specimens were prepared for study by sectioning the head portion which contains the pituitary and the thymus. The sections were stained with Haidenhain's iron alum-haematoxylin and eosine.

The glands were measured by taking the two dimensions from each section in the series and by counting the sections and multiplying by the thickness to get the other dimension. The average measurement of each dimension times the average of the other dimensions furnishes a means of comparing the glands for size. With an eye piece micrometer the longest diameter of the gland in any given section was measured. Then the micrometer was turned at right angles to the first dimension and the reading recorded.

In the later stages the desired measurements were obtained in a different manner. The specimens were too large to be readily sectioned through the head. Accordingly they were pinned on paraffin under a binocular microscope and the thymus was then exposed and dissected. Each gland was then put in a shallow dish and drawn with a camera lucida. The gland was first drawn showing the outline in a plane perpendicular to the shorter diameter. Then it was set up edgewise in a small groove cut in the paraffin so that the second drawing showed the outline in the plane of the shorter diameter. After

<sup>2</sup> Beard, J. 1900. *Anat. Anz.*, Bd. 18.

<sup>3</sup> Gudernatsch. 1914 *Am. Jour. Anat.*, vol. 15.

each drawing a scale was placed under the microscope and the scale of magnification for that particular drawing indicated on the card with the drawing. This furnished a check on the adjustment of the camera lucida.

The pituitary gland was taken from the same specimens as was the thymus. Some of the specimens were injured in manipulation and in those cases the measurements of the destroyed glands can not be given. The procedure in the measuring of the pituitary gland was the same as for the thymus until it was advisable to measure the dissected gland. In these stages the pituitary is much smaller than the thymus and harder to handle. The most satisfactory method was to dissect the whole brain and take it out of the specimen with the pituitary gland attached. The dura mater was carefully removed to expose the gland as much as possible. The gland was first drawn from its ventral aspect and then from the lateral aspect by the same instruments as were used in measuring the thymus.

The same methods were applied to the thyroidless specimens which had been prepared by Dr. Allen in the spring of 1916. These specimens were killed as needed over a period extending from a few days after removal in April 1916 to March 1917. The oldest operated tadpole was killed early in March. None of the thyroidless tadpoles metamorphosed into frogs and the comparisons in the later stages are between thyroidless tadpoles and normal frogs of about the same age as the experimental animals.

In determining the difference in size of the glands in the thyroidless and the control animals it is necessary to use a standard of comparison rather than the actual volumes because of the method adopted. The three dimensions obtained by the method outlined in the preceding paragraphs were multiplied together which gives the volume of a parallelopiped of those dimensions. Since conditions were uniform in the measuring the same relative error would apply to all. When the glands vary much from the average of the class to which they belong due to irregular contour the difficulty can be adjusted by reference to the drawings. Correction for body length was made by dividing the volume by the body length.

## MIGRATION OF THE THYMUS GLANDS

*Results*

In the course of this work it was found that the thymus glands of normal animals migrate in the process of metamorphosis. In normal tadpoles the thymus gland is found ventral and posterior to the eye and attached to the skull by connective tissue. In the metamorphosing frog as the head is taking on adult characters the thymus gland is found to have migrated posteriorly to a point just ventral to the tympanum. In the adult frog it is found in a mass of connective tissue under the depressor mandibuli muscle, thus coming to lie ventral and posterior to the tympanum. The thyroidless tadpoles still have the glands close to the eye and anterior to the tympanic membrane.

When the frog reaches the stage of sexual maturity, the thymus is flattened. This is due to its position between the muscles. In going from the tadpole to the recently metamorphosed frog stage it will be noticed in the drawings that there is sudden decrease in the size of the thymus glands and at the same time the glands become more globular. This is attributed to the loss of water from the tissues at metamorphosis. Reference to tables 1 and 2 will show that there is a decided shrinkage of the body at the time of metamorphosis, which is to be seen at a glance by any one who has reared them. It is not surprising that there should be shrinkage of the pituitary and thymus glands.

The pituitary and the thymus glands vary much in size and these differences can not always be explained by a corresponding difference in body length. This is strikingly shown by examination of the class of normal tadpoles, specimens 5 to 10, which were killed in July (table 2). Control 5 has a body length of 27 mm. and a pituitary gland represented by 58 as determined by the method explained above. Control 10 has a body length of 34 mm. and a pituitary gland represented by 224. This shows a development of the pituitary gland corresponding with the development of the tadpole.

TABLE 1  
*Measurements of controls*

SPECIMEN	TOTAL LENGTH	BODY LENGTH	PITUITARY ANTERIOR LOBE, SAGITTAL PLANE	PITUITARY TRANSVERSE	PITUITARY PERPENDICULAR IN SAGITTAL PLANE	RIGHT THYROID SAGITTAL	RIGHT THYROID TRANSVERSE	RIGHT THYROID PERPENDICULAR IN SAGITTAL PLANE	LEFT THYROID SAGITTAL	LEFT THYROID TRANSVERSE	LEFT THYROID PERPENDICULAR IN SAGITTAL PLANE
1	10.5		0.110	0.133	0.041	0.230	0.094	0.134	0.220	0.094	0.134
2	14.0		0.200	0.163	0.045	0.360	0.111	0.165	0.360	0.111	0.165
3	24.0		0.280	0.238	0.075	0.850	0.310	0.434	0.870	0.310	0.434
4			0.180	0.273	0.071	0.330	0.254	0.459	0.340	0.317	0.542
5	62.0	27.0	0.375	0.392	0.107	1.967	0.535	0.950	1.967	0.670	0.678
6	72.0	27.0	0.428	0.535	0.160	2.178	0.893	1.071	2.017	0.750	1.107
7	73.0	30.0	0.500	0.642	0.178	2.464	0.857	1.250	2.392	0.857	1.250
8	82.0	31.0	0.410	0.678	0.142	2.500	0.625	1.142	2.071	0.892	1.107
9	64.0	32.0	0.571	0.714	0.142	2.464	0.714	1.142	2.500	0.642	1.232
10	74.0	34.0	0.500	0.714	0.214	2.250	0.892	1.250	2.250	0.892	1.250
11		23.0				1.089	0.571	0.714	1.089	0.660	0.785
12		24.0				1.303	0.428	0.857	1.107	0.500	0.857
13		24.0	0.428	0.642	0.178	1.428	0.714	0.821	1.392	0.642	1.071
14		24.0	0.357	0.357	0.142	1.125	0.464	0.750	1.125	0.500	0.723
15		24.0				1.107	0.571	0.785	1.107	0.571	0.857
16		24.0	0.428	0.535	0.142	1.535	0.821	1.107	1.964	0.821	1.071
17		46.0	0.607	1.125	0.232						
18		46.0				1.857	0.250	0.714	1.948	0.232	0.767
19		48.0				1.535	0.178	0.857			
20		50.0	0.821	1.178	0.250	2.438	0.393	0.642	2.328	0.393	0.892
21		45.0	0.714	1.000	0.214				1.607	0.428	0.714
22		48.0	0.785	1.303	0.196						
23		50.0	0.785	1.250	0.214	1.964	0.214	0.928	1.821	0.250	0.857
24		51.0	0.875	1.142	0.250	2.357	0.285	0.857	2.392	0.285	0.857

*Measurements of thyroidless tadpoles. Sq. mm.*

1	10.5		0.060	0.154	0.041	0.170	0.104	0.138	0.160	0.104	0.138
2	13.0		0.090	0.188	0.065	0.180	0.178	0.216	0.180	0.178	0.216
3	24.0		0.280	0.192	0.074	0.760	0.236	0.255	0.800	0.255	0.360
4	85.0	36.0	0.569	1.020	0.300	1.458	0.341	0.900			
5	45.0	16.2	0.482	0.482	0.110						
6	97.0		1.125	1.000	0.410				3.196	0.428	1.071
7		33.0	0.892	1.017	0.457	1.892	0.446	0.875	2.857	0.446	0.946
8	123.0	43.0	1.017	1.232	0.321				3.250	0.535	1.446
9	76.0	34.0	0.607	0.821	0.357	2.678	0.482	1.178	3.142	0.446	1.196
10	102.0	40.0	0.875	1.375	0.303	2.750	0.410	0.842	2.785	0.410	0.835

TABLE 2  
*Average volumes of thymus and pituitary glands. Cu. mm.*

Specimen number	PITUITARY GLAND				THYMUS GLAND			
	Total length	Body length	Volume	Corrected volume	Right thymus volume	Left thymus volume	Combined volume	Corrected combined volume
Controls								
5	62	27.0	.015	.058	.999	.809	1.809	.670
6	72	27.0	.036	.135	2,083	1,674	3.757	1,391
7	73	30.0	.057	.190	2,639	2,562	5.201	1,733
8	82	31.0	.039	.127	1,784	2,044	3.829	1,235
9	64	32.0	.057	.180	2,011	1,977	3.988	1,234
10	74	34.0	.076	.224	2,508	2,508	5.017	1,475
11		23.0			.443	.563	1.075	.438
12		24.0			.530	.474	1.004	.418
13		24.0	.048	.203	.837	.957	1.794	.747
14		24.0	.018	.075	.391	.406	.797	.332
15		24.0			.496	.496	.992	.413
16		24.0	.032	.135	1,395	1,730	3.125	1,302
17		46.0	.158	.344				
18		46.0			.331	.344	.676	.147
19		48.0			.234	.234	.468	.097
20		50.0	.241	.483	.615	.816	1.431	.286
21		45.0	.154	.344	.491	.491	.982	.218
22		48.0	.200	.417				
23		50.0	.209	.419	.390	.390	.780	.156
24		51.0	.249	.489	.575	.584	1.159	.227
Thyroidless								
4	85	36.0	.174	.483	.447		.894	.248
5	45	16.2	.041	.254				
7		33.0	.313	.950	.693	1,205	1.899	.575
8	123	43.0	.402	.935		2,621	5.242	1,219
9	76	34.0	.177	.520	1,520		3.196	.940
10	102	40.0	.364	.911	.949	.953	1.902	.475

*Average volume of anterior lobe of the pituitary gland of:*

Controls

Normal tadpoles, Specimens 5 to 10..... .152

Young frogs, Specimens 13 to 16..... .138

Young sexually mature frogs, Specimens 17 to 24..... .416

Thyroidless tadpoles, *Thyroidless* 7 to 10..... .776



*Average volume of thymus glands of:*

## Controls

Normal tadpoles, Specimens 5 to 10.....	1.293
Young frogs, Specimens 11 to 16.....	.608
Young sexually mature frogs, Specimens 18 to 24.....	.182
Thyroidless tadpoles, <i>Thyroidless</i> 7 to 10.....	.802

Contrary to this, control 7, a 30 mm. tadpole has a pituitary gland represented by 190 while control 8, a 31 mm. tadpole has a pituitary gland represented by 127 (table 2). In this case the smaller tadpole has the larger pituitary gland thus showing individual variation. In these cases the thymus glands are larger in the animal containing the larger pituitary gland.

There are several reasons for this degree of variation. In the first place tadpoles reared in a natural environment show marked differences in rate of development even when derived from the same mass of eggs. When placed under artificial conditions in the laboratory these differences are accentuated. This is especially true of the later stages. Such differences are of influence in bringing about differences in the quantity of food, oxygenation, etc. This was accentuated by the fact that these tadpoles developed twisted tails as described in Dr. Allen's paper.<sup>4</sup> As regards the thymus gland it is well known that this is an extremely variable structure in man. Further study may show the same thing to be true in the frog. The most extreme variation in these glands as seen in the controls is found at the period immediately after metamorphosis. This is to be expected because of the sudden and profound changes that most of the organs of the body have undergone. A slight variation in the rate of development will bring about marked individual differences in the volume of these glands.

With such a small number of specimens available it is not claimed that the results of this work are quantitatively accurate within a wide range of probable error; but the results seem sufficiently consistent and definite to justify the conclusions drawn.

In some specimens one thymus gland is larger than the other of the pair. A case of this kind is found in control 6 (table

<sup>4</sup> Allen, B. M. 1917 Jour. Exp. Zool., vol. 24.

2). For the most part the pair of thymus glands approximate each other in size. Thyroidless tadpole 7 shows a marked difference in the size of the two thymus glands. This variation is thus found in both thyroidless and control specimens.

COMPARISONS OF THE ANTERIOR LOBE OF THE PITUITARY GLAND  
AND OF THE THYMUS GLANDS IN YOUNG TADPOLES

In comparison there is nothing striking in the 10.5, 13, and 24 mm. total length (table 1), respectively except in the growth of the normal thymus. Both the pituitary gland and the thymus are slightly larger in the 10.5 mm. control tadpole. The 13 mm. thyroidless tadpole has somewhat larger glands than the 14 mm. control tadpole. The 24 mm. control tadpole has better developed glands than the 24 mm. thyroidless tadpole. Here it is noticed that the normal thymus has made a markedly greater increase than that of the thyroidless tadpole. This proves nothing at this stage but it gives a hint as to the further growth of the thymus gland in the larval frog.

COMPARISONS OF THE ANTERIOR LOBE OF THE PITUITARY GLAND  
AND OF THE THYMUS GLANDS IN METAMORPHOSING NOR-  
MAL TADPOLES AND THYROIDLESS TADPOLES OF COMPAR-  
ABLE AGE

The period of normal metamorphosis for *Rana pipiens* extends from June to August, depending on the time at which the eggs were laid, the season, the food, etc. The thyroidless tadpole 4 (table 2) was killed September 25, 1916 and had a body length of 36 mm. This specimen was killed soon after the time when normal metamorphosis took place. The largest of the metamorphosed control tadpoles, No. 10 (table 2) had a body length of 34 mm. The average body length of these controls was 30.16 mm. The thyroidless tadpole (table 2, no. 4) killed September 25, had a pituitary gland which with body length correction was represented by 483. The average of the pituitary glands of the normal metamorphosing tadpoles on the same basis was 152. The pituitary gland of the thyroidless tadpole was thus very much larger than the average for the

metamorphosing normal tadpoles. This shows that the pituitary gland of the thyroidless specimen has made a marked increase over the normal pituitary gland. The portion of the pituitary glands in question is the anterior lobe. Thyroidless tadpole 5 (table 2), with a body length of 16.2 mm. was abnormally small. The pituitary gland was smaller than that of the September 25 (table 2, no. 4) thyroidless specimen. When the pituitary gland of this specimen is considered with body length correction as were the glands of the control and the thyroidless tadpoles it has a relative size of 254. Even in this small thyroidless tadpole the pituitary gland is proportionally larger than in the control tadpoles.

The thymus gland of the tadpole killed September 25 (no. 4, table 2), had a relative volume with body length correction of 248. The thymus glands of the control tadpoles were much larger and the average for the class was 1293. This shows that during the period of metamorphosis the thymus gland is increased very much. The thymus gland of the control tadpole is therefore much larger than that of the thyroidless tadpole which is slightly older.

The striking thing shown in this stage is that the pituitary gland of the thyroidless tadpole is much larger than that of the control tadpoles while the thymus glands of the latter are a great deal larger than those of the thyroidless tadpole. These differences are clearly shown on the graphs. The average size of the pituitary gland in recently metamorphosed frogs was represented by 138. This shows that the pituitary gland is smaller than in the thyroidless tadpole killed September 25 (table 2 and chart 1).

The average size of the thymus glands in recently metamorphosed frogs was represented by 608, (table 2). The thymus gland of the thyroidless tadpole killed September 25 (table 2, no. 4), two months later had a value of 248. This shows that the recently metamorphosed frog has larger glands than the thyroidless tadpole killed September 25; but smaller than those of normal tadpoles during metamorphosis (table 2 and chart 2).

The thyroidless tadpole has a larger pituitary gland than the recently metamorphosed control frog but the metamorphosed frog has larger thymus glands. The difference is not so great as in the case of the normal tadpoles.

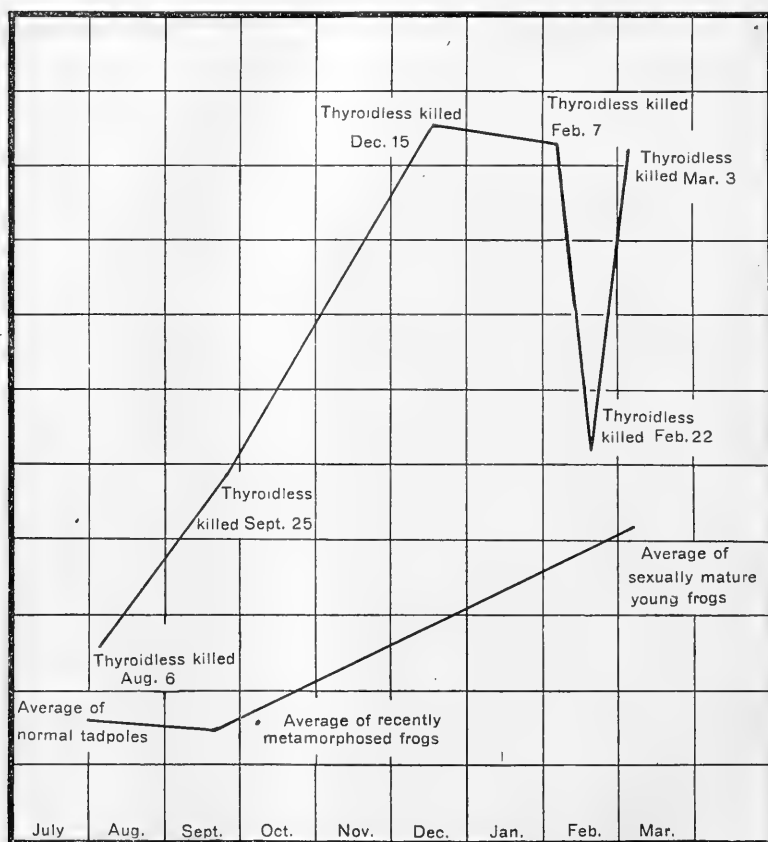


Chart 1. Growth curves of the pituitary gland with body length corrections.

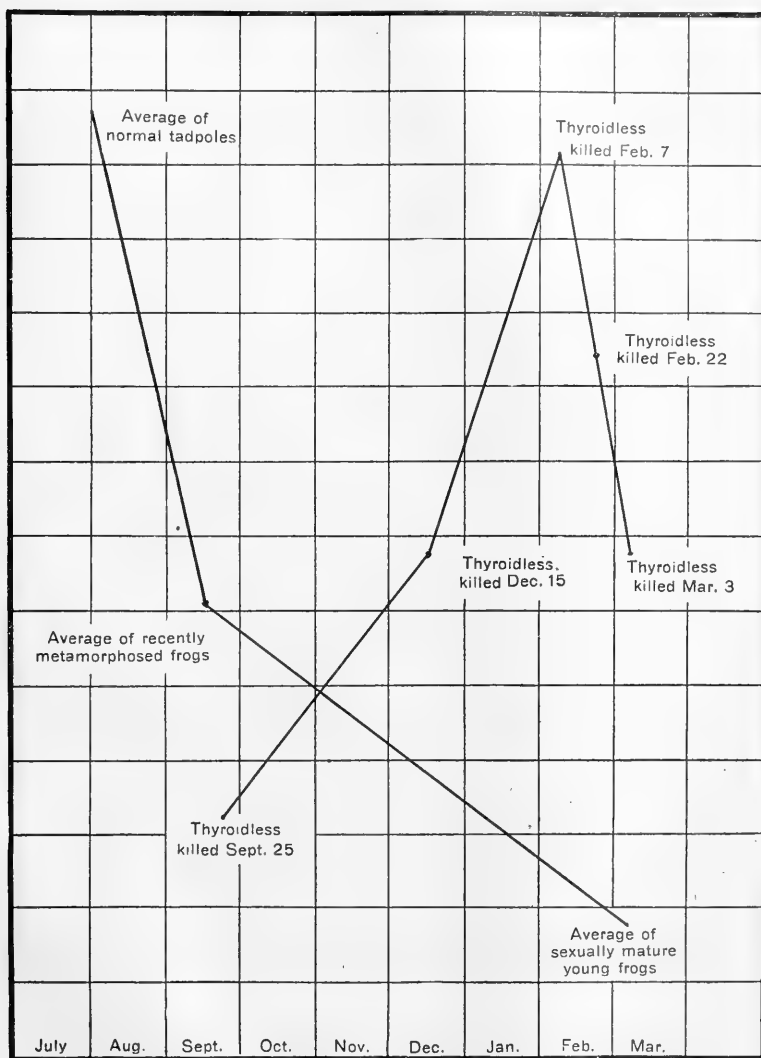


Chart 2. Growth curves of the thymus gland with body length corrections.

COMPARISONS OF THE ANTERIOR LOBE OF THE PITUITARY GLAND  
AND OF THE THYMUS GLANDS IN YOUNG SEXUALLY MATURE  
FROGS AND THYROIDLESS TADPOLES KILLED BETWEEN DE-  
CEMBER 15, 1915 AND MARCH 3, 1917

The thyroidless tadpole 7, table 2, killed December 15, 1916 had a body length of 33 mm. The pituitary gland was represented by 950 and the thymus gland by 575.

The thyroidless tadpole 8, table 2, killed February 7, 1917, had a body length of 43 mm. and was the largest of the thyroidless specimens. The pituitary gland was represented by 935 and the thymus gland by 1219 on the relative standard. The thyroidless tadpole 9, killed February 22, 1917, had a body length of 34 mm. The pituitary gland was represented by 520 and the thymus gland by 940, table 2. The thyroidless tadpole 10, table 2, killed March 3, 1917, had a body length of 40 mm. The pituitary gland was represented by 911 and the thymus gland by 475. The pituitary gland of this specimen was larger than that of the February 22, table 2, no. 9, specimen but smaller than of the February 7, table 2, no. 8, thyroidless tadpole. The thymus glands of this specimen were smaller than those of the other two thyroidless specimens killed a little earlier. There are not sufficient grounds however to say that the thymus glands are degenerating.

The controls for these older thyroidless tadpoles were a number of young sexually mature frogs that metamorphosed the preceding summer and were killed. February 18 to March 4. The average volume of the pituitary gland for this class of controls was 416, table 2. The pituitary gland in each of the four oldest thyroidless specimens was larger than in the controls.

The pituitary glands of the four oldest thyroidless tadpoles are represented by 950, 935, 520 and 911 respectively, table 2. It is apparent here that the pituitary gland in the thyroidless tadpoles grew larger than did the pituitary in young sexually mature frogs. This was especially noticeable when corrections were made for relative body length. It is remarkable, for the thyroidless specimens were still in the tadpole stage while the frogs had metamorphosed the preceding summer.

The average of the thymus glands for the young frog controls was 182, table 2. This volume varied in the different specimens from 110 to 286. The thymus glands of each of the three oldest thyroidless tadpoles is represented by 575, 1219, 940, and 475 respectively (tables 2, 8, 9, and 10). It is clear that in none of the young sexually mature frogs were the thymus glands as large as in the four oldest thyroidless tadpoles. The thymus gland of the young sexually mature control frogs is much smaller than that of the recently metamorphosed control frogs and the control tadpole has larger thymus glands than the recently metamorphosed frogs. The thymus degenerates as maturity is reached in most forms and this is what is happening here. In the case of the thyroidless tadpoles this degeneration has not taken place and instead the thymus gland has developed more and has grown during a longer period of time.

It is interesting to note here that the thyroidless tadpole killed February 7, table 2, no. 8, has well developed thymus glands in spite of the fact that the tadpole is sexually mature with many live sperm. The thymus gland has persisted after sexual maturity was reached.

#### THE GRAPHICAL REPRESENTATION OF THE VOLUMES

The number of thyroidless tadpoles used as a basis for the graphs described below was so small that the curves should be accepted as showing general features and not as showing the accurate details of growth. The volumes of the pituitary glands in both the thyroidless and the normal tadpoles do not vary much until the period of metamorphosis (table 1). The line for the thyroidless tadpoles remains high until the February 22 specimen is reached when it comes down only to go up again for the March 3 thyroidless tadpole (chart 1). The line for the control specimens comes up gradually until the young sexually mature frog stage is reached. The great deviation in the volume of the pituitary gland of the thyroidless tadpole killed February 22, table 2, no. 9, is probably due to individual variation and has no great significance.

The line of growth of the thymus glands in the thyroidless specimens mounts rapidly after the September 25 specimen is reached. It reaches its greatest height in the February 7 specimen and then drops. The sudden decline might mean degeneration but on account of the few thyroidless tadpoles observed it could not be determined.

The line for the normal control mounts very high during the period of metamorphosis. It then comes down sharply to the recently metamorphosed frog stage and slopes off gradually to the young sexually mature frog stage.

#### SUMMARY

1. The pituitary gland continues to develop when the thyroid gland is extirpated and the anterior lobe reaches a larger size actually and relatively than in normal specimens. In most cases it is larger than the pituitary gland of the corresponding control even without reference to body length. It is at its largest in proportion to body length in thyroidless tadpoles that are of the same age as young sexually mature frogs.

2. The thymus gland develops when the thyroid gland is extirpated. The thymus glands of normal controls are larger during the period of metamorphosis and immediately following than at any other period of the frog's life.

3. The young sexually mature frogs have smaller thymus glands than do thyroidless tadpoles of the same age.

4. The thymus glands of thyroidless tadpoles do not migrate to the position in which the thymus gland is found in adult frogs.

5. The thymus gland of the thyroidless tadpole does not degenerate like that of a frog that develops normally.



## PLATE 1

Magnification 12

*Pituitary glands of thyroidless tadpoles*

- 1 Pituitary gland of thyroidless tadpole 5
- 2 Pituitary gland of thyroidless tadpole 6
- 3 Pituitary gland of thyroidless tadpole 7
- 4 Pituitary gland of thyroidless tadpole 8
- 5 Pituitary gland of thyroidless tadpole 9
- 6 Pituitary gland of thyroidless tadpole 10

*Pituitary glands of normal tadpoles*

- 7 Pituitary gland of tadpole control 5
- 8 Pituitary gland of tadpole control 6
- 9 Pituitary gland of tadpole control 7
- 10 Pituitary gland of tadpole control 8
- 11 Pituitary gland of tadpole control 9
- 12 Pituitary gland of tadpole control 10

*Pituitary glands of recently metamorphosed frogs*

- 13 Pituitary of control frog 13
- 14 Pituitary of control frog 14
- 15 Pituitary of control frog 16

*Pituitary glands of sexually mature young frogs*

- 16 Pituitary gland of control frog 17
- 17 Pituitary gland of control frog 20
- 18 Pituitary gland of control frog 21
- 19 Pituitary gland of control frog 22
- 20 Pituitary gland of control frog 23
- 21 Pituitary gland of control frog 24

*Thymus glands of thyroidless tadpoles*

- |                                      |                                       |
|--------------------------------------|---------------------------------------|
| 22 Left thymus gland, thyroidless 6  |                                       |
| 23 Left thymus gland.                | 24 Right thymus gland, thyroidless 7  |
| 25 Right thymus gland, thyroidless 8 |                                       |
| 26 Left thymus gland.                | 27 Right thymus gland, thyroidless 9  |
| 28 Left thymus gland.                | 29 Right thymus gland, thyroidless 10 |

*Thymus glands of normal tadpoles*

- |                       |                                   |
|-----------------------|-----------------------------------|
| 30 Left thymus gland. | 31 Right thymus gland, control 5  |
| 32 Left thymus gland. | 33 Right thymus gland, control 6  |
| 34 Left thymus gland. | 35 Right thymus gland, control 7  |
| 36 Left thymus gland. | 37 Right thymus gland, control 8  |
| 38 Left thymus gland. | 39 Right thymus gland, control 9  |
| 40 Left thymus gland. | 41 Right thymus gland, control 10 |

*Thymus glands of recently metamorphosed frogs*

42	Left thymus gland.	43	Right thymus gland, control 11
44	Left thymus gland.	45	Right thymus gland, control 12
46	Left thymus gland.	47	Right thymus gland, control 13
48	Left thymus gland.	49	Right thymus gland, control 14
50	Left thymus gland.	51	Right thymus gland, control 15
52	Left thymus gland.	53	Right thymus gland, control 16

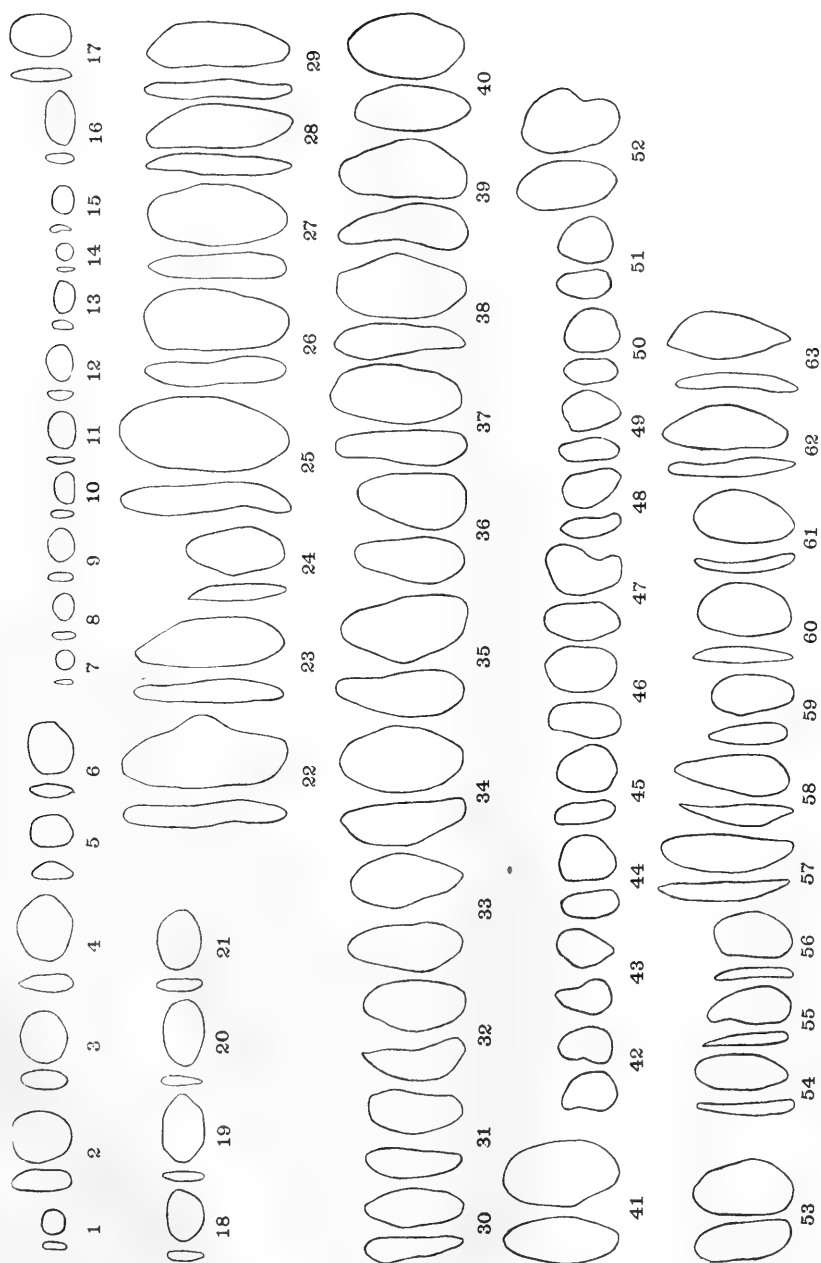
*Thymus glands of sexually mature young frogs*

54	Left thymus gland.	55	Right thymus gland, control 18
		56	Right thymus gland, control 19
57	Left thymus gland.	58	Right thymus gland, control 20
59	Left thymus gland.		control 21
60	Left thymus gland.	61	Right thymus gland, control 23
62	Left thymus gland.	63	Right thymus gland, control 24

# EFFECT OF EXTIRPATION OF THYROID

JAMES B. ROGERS

PLATE I





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